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16. ABSTRACT

The procedures for culturing marine polychaetous annelids from egg to egg under laboratory conditions were described. A manual was prepared detailing the procedures used in culturing 12 species of polychaetes. The polychaetes which have been successfully cultured and the number of cycles completed in the laboratory are: Neanthes arenaceodentata (50+), Capitella capitata (50+), Ctenodrilus serratus (50+), Ophryotrocha diadema (50+), O. puerilis (20+), Dinophilus sp. (50+), Dexiospira brasiliensis (3), Polydora ligni (3), Boccardia proboscidea (3), Cirriformia luxuriosa (1), C. Spirabrancha (1), and Halosydna johnsoni (1).

The effects of heavy metals and the water soluble fractions of petroleum hydrocarbons were measured over 96 hours, 28 days, and with some of the toxicants, over a complete reproductive cycle for some of these species of polychaetes. Mercury and copper were the most toxic of the six metals tested and cadmium was the least toxic. The 28-day LC_{50} was less than the 96-hour value in most experiments. Larval stages were more sensitive than the adults to heavy metals. Dexiospira was the most sensitive species and Cirriformia luxuriosa was the most tolerant. Suppression of reproduction occurred with each species studied when exposed to heavy metals; the concentrations at which this occurred was less than the 28-day LC_{50}.

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THE EFFECT OF DIFFERENT POLLUTANTS ON ECOLOGICALLY IMPORTANT POLYCHAETE WORMS

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FOREWORD

The Environmental Research Laboratory of the U.S. Environmental Protection Agency is located on the shore of Narragansett Bay, Rhode Island. In order to assure the protection of marine resources, the laboratory is charged with providing a scientifically sound basis for Agency decisions on the environmental safety of various uses of marine systems. To a great extent, this requires research on the tolerance of marine organisms and their life stages as well as of ecosystems to many forms of pollution stress. In addition, a knowledge of pollutant transport and fate is needed.

This report describes the results of a 3.5 year study to establish laboratory colonies of polychaetous annelids and to measure the effects of heavy metals and petroleum hydrocarbons on these organisms through a complete life cycle. This report covers the period of time from late 1972 through June 1976. Certain phases of the investigations have been updated since the termination of the grant to reflect additional data which has been accumulated since June, 1976.

ABSTRACT

The procedures for culturing marine polychaetous annelids from egg to egg under laboratory conditions were described. A manual was prepared detailing the procedures used in culturing 12 species of polychaetes. The polychaetes which have been successfully cultured and the number of cycles completed in the laboratory are: *Neanthes arenaceodentata* (50+), *Capitella capitata* (50+), *Ctenodrilus serratus* (50+), *Ophryotrocha diadema* (50+), *O. puerilis* (20+), *Dinophilus sp.* (50+), *Dexiospira brasiliensis* (3), *Polydora ligni* (3), *Boccardia proboscidea* (3), *Cirriformia luxuriosa* (1), *C. spirabrancha* (1), and *Halosydna johnsoni* (1).

The effects of heavy metals and the water soluble fractions of petroleum hydrocarbons were measured over 96 hours, 28 days, and with some of the toxicants, over a complete reproductive cycle for some of these species of polychaetes. Mercury and copper were the most toxic of the six metals tested and cadmium was the least toxic. The 28-day LC_{50} was less than the 96-hour value in most experiments. Larval stages were more sensitive than the adults to heavy metals. *Dexiospira* was the most sensitive species and *Cirriformia luxuriosa* was the most tolerant. Suppression of reproduction occurred with each species studied when exposed to heavy metals; the concentrations at which this occurred was less than the 28-day LC_{50}.

The water soluble fraction of a refined oil was more toxic than a crude oil. *Capitella* was the most tolerant species to the oils and *Ophryotrocha diadema* was the most sensitive one tested. Suppression of reproduction was observed for both *Ctenodrilus* and *Ophryotrocha diadema*.

An interlaboratory calibration experiment using *Capitella* was described and the publication resulting from this study has been published elsewhere.

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SECTION 1

INTRODUCTION

Polychaetous annelids are segmented worms that are present in all oceanic regions of the world and in all habitats. A few species have been found in fresh water, usually upstream from estuaries. Polychaetes are abundant in the intertidal environments, especially in sediments consisting of silts and clays. The subtidal benthos is their preferred habitat since they are most numerous here, both in terms of numbers of species and total specimens. Polychaetes are also the most numerous macrofaunal component of the soft bottom community; the percentage of polychaete species and specimens present is generally between 30 and 50 regardless of geographical region or depth (Knox, 1977). Polychaetes play an important role in the movement of sediments in much the same manner as earthworms do on land. Polychaetes also play an important role in providing food for birds and fish (Reish and Ware, 1976).

The study of marine pollution can be divided into two areas, field studies and laboratory studies. Laboratory studies have generally focused on the toxic effect of a single compound on an economically important organism such as fish, with the results generally expressed as a 96-hour LC_{50}, the concentration at which 50 percent of the organisms die in 96 hours. Application factors, generally 0.01, were applied to this figure to estimate the long-term effect level. With the discovery of the effects of DDT on reproduction in birds, it became apparent that in order to obtain a clearer understanding of the effects of a toxicant, toxicity tests must be conducted over an entire life cycle of the organism, including the effects on its offspring. Because of their importance in marine environments, polychaetes were a logical choice with which to study long-term effects under laboratory conditions.

The majority of marine invertebrates, including polychaetes, had never been cultured previously in the laboratory; those studies that had been done with invertebrates were pursued only through larval stages. For the most part, mass culture methods had never been considered for marine organisms, except possibly those of economic importance. In order to measure the long-term effect of a pollutant on the reproduction, fertilization, larval development, settlement, growth, and reproduction of the F_1 generation, it is necessary to have large quantities of specimens available on a routine basis. Therefore, the two primary objectives of this study were as follows:

1. To develop techniques for culturing several species of polychaetes through their life cycle under laboratory conditions, particularly for those species that could reproduce on a more or less continuous basis under laboratory conditions.

2. To study the short-term and long-term effects of various toxicants, such as heavy metals and petrochemicals, on those polychaete species that were in laboratory culture, with emphasis on studying the effect of these toxicants on reproduction as measured by the number of eggs or offspring produced.

SECTION 2

LABORATORY CULTURE PROCEDURES WITH POLYCHAETOUS ANNELIDS

Prior to the initiation of this study, the author had two species of polychaetes under laboratory culture; these were *Neanthes arenaceodentata* and *Capitella capitata*. The *N. arenaceodentata* colony was established in 1964 and had undergone about 35-40 generations in the laboratory by the initiation of this study in late 1972. The original colony was established from six worms collected from Los Angeles Harbor. The *C. capitata* colony was started from a single female collected from Los Angeles Harbor in 1968. *C. capitata* has a faster life cycle and had undergone about 40 generations by the initiation of this study.

A total of twelve species, including *N. arenaceodentata* and *C. capitata*, were under laboratory culture, of which eleven had gone through at least one complete life cycle in the laboratory and six had completed at least twenty life cycles. *Cirriformia luxuriosa* was the largest worm and after two years had not yet completed its life cycle. Those species that were cultured in the laboratory during the course of this research are summarized in Table 1. Additional data included in Table 1 were the number of life cycles each species had completed, the length of the life cycle, and the status of the colony in 1979. A manual of culture techniques for twelve species of polychaetes has been included with this report (Appendix A-I) and can be found elsewhere (Reish, 1976). Additional information concerning the culture techniques and the usefulness of the particular species in bioassay studies is noted below.

N. arenaceodentata (Appendix A). This species is a convenient laboratory bioassay organism. The adults are sufficient size and weight (2-4 cm; 50-100 mg wet weight) to permit handling with ease and to make body burden analysis for toxicants possible. The life cycle, of three to four months duration at 20°C, is of sufficient length to permit long-term studies. Short-term tests can be conducted with either juveniles or adults. Survival of controls is almost always 100 percent, and death is clearly defined. The female lays about 150-400 large eggs (500 μ in diameter) in the male's tube and then she dies. There is no planktonic larval stage. The male cares for the young until they leave in about one month at the 18-21 segmented stage. The data obtained in reproductive tests provide information on whether or not *N. arenaceodentata* will reproduce at a particular concentration and the number of eggs laid (or juveniles emerged) per female. The data can be subjected to a variety of statistical tests to determine if the results are of significance (American Public Health, 1976).

N. arenaceodentata is the species favored by the author for bioassay work. An established colony of 3,000-5,000 specimens requires about 30-40 hours per month to maintain. A further advantage of working with this species is the ease in shipping live specimens to other geographical regions. Five adult specimens are placed in a 11 x 23 cm plastic bag together with 24 ml of seawater and a small amount of resoaked *Enteromorpha*. The bags are then placed in a box and sent by air mail. Successful shipments (numbering about 80 as of December 1979) of *N. arenaceodentata* have been sent to laboratories located on the east coast of the United States, in the Gulf States, on the Pacific coast, and in Canada.

C. capitata (Appendix B). The life cycle of this species is 30-40 days at 20°C. The female lays about 200-400 eggs in her tube, which she incubates for four to five days. The young larvae leave the tube of the parent and may swim for a few hours before settlement and metamorphosis. The number of eggs laid by the female makes it possible to obtain data on the effects of a toxicant on reproduction and number of eggs produced. The induction of abnormal larvae (bifurcated posterior ends) is a method of measuring sublethal effects (Reish, and others, 1974; Reish, 1977). Short-term bioassays can be conducted with both trochophores and adults. Survival of controls has been at least 90 percent, and with practice, death of specimens can be readily distinguished. Long-term bioassays can be initiated with young *Capitella* and carried out through one or more life cycles. *Capitella* is an easily cultured species; a colony of 3,000-5,000 worms requires about 15 hours per month to maintain. Living specimens have been shipped by air mail to the east coast of the United States and to Texas, Canada, and France with a good survival rate. Approximately 25-40 specimens are placed in a 11 x 23 cm plastic bag together with 25 ml of seawater and a small amount of resoaked *Enteromorpha*. The bags are then placed in a box and mailed.

Ctenodrilus serratus (Appendix C). *Ctenodrilus* is a small polychaete that reproduces asexually by transverse fission in 12-15 days at 20°C. All specimens were derived from a single worm collected in Los Angeles Harbor in 1972. It is a convenient species with which to conduct toxicity tests on reproduction since the life cycle is completed in less than a month. Survival of control specimens has been at least 95 percent. A large colony can be cultured in five 3.78 ℓ aquaria with only three to five hours per month required for maintenance.

Ophryotrocha diadema (Appendix D). This species was described by Akesson (1976) from specimens collected in Los Angeles Harbor in 1972. The colony has been maintained since this original collection. *O. diadema* is a protandic hermaphrodite that completes its life cycle in 20-25 days at 20°C. Bioassay procedures similar to those described for *Ctenodrilus* are used. Survival of control specimens over a 96-hour experiment has been at least 90 percent. A colony of 3,000-5,000 specimens requires about 12-15 3.78 ℓ aquaria and about ten hours per month to maintain.

Ophryotrocha puerilis (Appendix D). This species has been cultured in Sweden by Akesson for several years. The present colony was established from specimens brought to Long Beach by one of Akesson's colleagues in 1974. The methods of culturing *O. puerilis* are similar to those of *O. diadema*, but the life cycle is shorter in the former species, hence its more frequent use in bioassays by the author.

Dexiospira brasiliensis (Appendix E). This is one of the many common small spirorbid-type serpulid polychaetes that builds a tightly coiled calcareous tube. Specimens were collected from the surface of mussels or algae in Alamitos Bay. *D. brasiliensis* broods a small number of embryos in its operculum. The number of embryos or the amount of calcareous deposition in the tube can be related to the concentration of toxicant. While this species requires only a month to complete its life cycle, it requires several hours per week to maintain a small colony of less than 1,000 since the phytoplanktonic organism *Dunaliella* must also be cultured to provide food for *D. brasiliensis*.

Polydora ligni (Appendix F). This is a commonly encountered species in southern California waters, both as a bottom-dwelling adult or as a planktonic larvae. Both stages were collected from nearby Bolsa Chica. The larvae have been used for 96-hour bioassays, but they are not suitable for longer tests because of the low survival rate in culture. The number of eggs per capsule and the number of capsules per spawning period may be convenient sublethal parameters to study. Both larvae and adults require living *Dunaliella* as food. Several hours per week are required to maintain a colony of about 400 because of the time required to culture the *Dunaliella* and for feeding.

Boccardia proboscidea (Appendix F). This species of spionid polychaete is encountered less frequently in southern California waters than *Polydora ligni*. All details concerning culturing and conducting bioassays with this species are identical to those given for *Polydora ligni* in Appendix F.

Dinophilus sp. (Appendix G). This is a small species of an archiannelid polychaete that appeared in some of the stock colonies of *Neanthes* and *Capitella*. Presumably it was present in the seawater transported to the campus in 1973 by a tank truck from Marineland of the Pacific. It probably represents an undescribed species. It is about the same size as *Ophryotrocha* but can be distinguished by the presence of one anal cirrus rather than two as in *Ophryotrocha*. This minute species has about a five-day life cycle, therefore it is not a suitable test organism for a 96-hour bioassay. However, *Dinophilus* is especially useful for studying the effects of a toxicant over two or three life cycles.

Cirriformia luxuriosa and *Cirriformia spirabrancha* (Appendix H). These two closely related species are several centimeters in length and require one to two years to complete their life cycle. Culture methods are identical for both species. Specimens of *C. luxuriosa* were collected from the mussel community in Alamitos Bay, and *C. spirabrancha* were collected from intertidal muddy sand flats in Alamitos Bay in Long Beach. Large numbers of specimens may be kept in various sized aquaria, but for best results a five cm layer of coarse sand, which prevents the worms from becoming tangled with one another, should be placed in the aquaria. This is especially important since both species have numerous long filiform gills arising from the notopodial region along the entire length of the worm, that makes the worms difficult to separate. Adults are fed a mixture of finely chopped, dried *Enteromorpha* and commercially prepared fish food flakes. A stock colony of 1,000 adults of either species requires five to eight hours of maintenance per week. Since these two species are large in size and have a long life cycle, they would be excellent benthic organisms to conduct long-term bioassays and determine body burden levels of a toxicant.

Halosydna johnsoni (Appendix I). One complete life cycle has been carried out in the laboratory, the first such instance in the largest family of polychaetes, the Polynoidae. Specimens were collected from the mussel community in Alamitos Bay in Long Beach. Each female lays up to 100,000 eggs, which makes this species particularly well suited for larval testing. The adults require a considerable amount of individual attention since they must be kept in separate containers because of their cannibalistic tendencies. Adults can be maintained in a small glass tubing. Frozen brine shrimp are fed to each specimen twice a week. This species lives much better at 17°C than at 20°C. About 15 hours a week are required to maintain about 500 specimens.

The feasibility of rearing polychaetes in large numbers under laboratory conditions for use in short-term or long-term bioassays over at least one complete life history has been demonstrated. Large populations of up to ten species of polychaetes can be maintained by one full-time laboratory assistant. However, it is advantageous to have more than one technician trained on culture techniques in the event of illness, vacations, etc. Many of these species have been successfully transported to other laboratories for use in various types of investigations.

SECTION 3

THE EFFECTS OF HEAVY METALS ON POLYCHAETOUS ANNELIDS

The effects of six heavy metals were studied, at least in part, on nine different species of polychaetes (Table 2). Metals included were cadmium ($CdCl_2$), chromium (CrO_3), copper ($CuSO_4 \cdot 5H_2O$), mercury $HgCl_2$), lead ($Pb(CH_3COO)_2 \cdot 3H_2O$), and zinc ($ZnSO_4 \cdot 7H_2O$). It was necessary to add sodium citrate as a chelating agent to the copper, zinc, and lead solutions; a second control was used that contained this agent at the highest concentration used. Data generated included 96-hour and 28-day LC_{50} values (Table 2) and effect on reproduction as measured by the number of offspring produced. The results of much of this work dealing with the effects of heavy metals on polychaetes have been published elsewhere (Appendix K).

96-HOUR AND 28-DAY LC_{50} DATA

The results of these experiments can be summarized as follows:

1. Comparisons of the 28-day LC_{50} to the 96-hour LC_{50} data indicated that within a species most of the 28-day values were less than the 96-hour values. The 96-hour and 28-day LC_{50} data for copper and mercury were previously reported for most of the copper species (Reish, and others, 1976). Similar 96-hour and 28-day LC_{50} values for the species exposed to mercury may be attributable to the static exposure conditions since mercury is a volatile element. Thus, the mercury concentrations at 28 days were most likely less than at 96-hours. No explanation is offered to account for the similar results obtained with the copper experiments.

2. In the limited number of experiments conducted with larvae, the larval stage was more sensitive than the adult to heavy metals (Reish, 1977, 1978; Reish and Carr, 1978). Trochophores were more sensitive than the adults to cadmium, copper, lead, mercury, and zinc but were more tolerant to chromium. Juvenile *Neanthes* were more sensitive than adults to all metals except cadmium and chromium. Trochophores of *Halosydna* were more sensitive to chromium, copper, and zinc, but in contrast, were less sensitive to mercury (Table 2).

3. Mercury and copper were the most toxic of the six metals to all species tested. Cadmium was the least toxic (Reish and others, 1976).

4. While a complete comparison is impossible since not all the experiments indicated in Table 2 were completed, some generalities can be made on the relative sensitivity of these species of polychaetes to the metals tested.

    a. *Dexiospira* was the most sensitive species in the two instances where data are available (Cu, Zn); the 28-day LC_{50} data were about one order of magnitude lower than those values for the other species (Table 2).

    b. *Cirriformia luxuriosa* was the most tolerant species for which data are available (Cu, Zn, Cd) (Table 2).

    c. While the relative sensitivities of the species were generally constant, one noteworthy exception was observed, *Halosydna* was very tolerant to five of the six metals tested but very sensitive to copper.

REPRODUCTION

The effect of these six metals on reproduction was studied in detail with *Ctenodrilus* and *Ophryotrocha* which possess a short life cycle, and to a limited extent with *Capitella* and *Neanthes* (Table 3). The data included in this table are the highest concentration at which reproduction occurred and the concentration at which a statistically significant suppression of reproduction was noted. The findings can be summarized as follows:

1. The effect of these six metals on the reproduction of *Ophryotrocha* and *Ctenodrilus* is in general similar; however, *Ophryotrocha* tends to be the more sensitive species except to chromium. *Ophryotrocha* was much more sensitive to zinc than *Ctenodrilus* (Reish, 1978; Reish and Carr, 1978).

2. Supression of reproduction in *Capitella* occurred at a lower concentration of copper (0.05 mg/l) than in either *Ophryotrocha* or *Ctenodrilus* (0.1 mg/l). Abnormal larvae were induced in *Capitella* at an even lower concentration, 0.01 mg/l (Reish and others, 1974).

3. Reproductive suppression in *Neanthes* occurred at a very low concentration of chromium, .0125 mg/l (Oshida and others, 1976).

4. Comparison of the 96-hour LC_{50} data to the concentration at which reproductive suppression occurred in *Ctenodrilus* and *Ophryotrocha* is of special significance (Tables 2 and 3). The concentration at which reproductive suppression occurred was from one to two orders of magnitude less than the 96-hour LC_{50} for *Ophryotrocha*. In *Ctenodrilus* the range in which reproductive suppression occurred was from slightly lower than the LC_{50} to two orders of magnitude different (Reish and Carr, 1978). With mercury, however, the concentration for suppression for both species was approximately the same as the 96-hour LC_{50}. These results with mercury may be attributable to the static exposure conditions since mercury is a

volatile element. However, no explanation is offered to account for similar results with copper.

5. For each metal, there was a sublethal concentration at which reproduction occurred but was significantly reduced. Also, in the case of *Capitella* there was a lower concentration (with chromium, copper, and zinc) at which the reproductive rate was not affected but abnormal larvae were induced (Reish and others, 1974; Reish, 1977).

Interestingly, the magnitude of difference between the 96-hour LC50 and that concentration at which there was no significant suppression of reproduction was less than one for animals exposed to mercury and copper but was greater than one to cadmium, chromium, lead, and zinc. As stated above the similar results in tests with mercury may be attributed to the volatile nature of the element since the solution was not renewed during the experiment. The action of cadmium, chromium, lead, and zinc may take a longer period of time to cause an effect, or the animal has an ability to excrete at least some of the metal.

In summary, this study has shown that it is feasible to conduct toxicity studies with polychaetes with reproduction as the parameter that is measured. Since reproduction is obviously necessary for the survival of the species, to be able to determine at what levels the organism can or cannot reproduce is of vital importance to assess the effects of pollution.

SECTION 4
THE EFFECTS OF PETROLEUM HYDROCARBONS ON POLYCHAETOUS ANNELIDS

The toxicty of water-soluble fractions of No. 2 fuel oil and South Louisiana crude oil to five species of polychaetes was determined. These species were *Capitella capitata, Ctenodrilus serratus, Ophryotrocha diadema, Dexiospira brasiliensis,* and *Cirriformia spirabrancha.* Toxicity was reported as 96-hour and 14-day LC_{50} values for these five species (Table 4). In addition, the effect of these two oils on reproduction was measured for *Ctenodrilus serratus* and *Ophryotrocha diadema* (Table 5). The results of these experiments can be summarized as follows: (see also Carr and Reish, 1977.)

1. The water-soluble fraction of No. 2 fuel oil (a refined oil) was more toxic to all species of polychaetes, except *Cirriformia* sp., at both 96 hours and 14 days than South Louisiana crude.

2. In many of the experiments with both No. 2 fuel oil and South Louisiana crude the 96-hour testing period was an insufficient length of time to show any toxic effect to these species of polychaetes.

3. *Capitella* was the most tolerant species tested to both oils (identical to *Cirriformia* sp. exposed to No. 2 fuel oil) and *Ophryotrocha* sp. was the most sensitive species tested.

4. A significant suppression of reproduction was observed for *Ctenodrilus* and *Ophryotrocha* exposed to both oils. The percent concentration of oil at which reproductive suppression occurred was always less than the 14-day LC_{50} percent concentration level.

In summary, as in the case with the heavy metals study, long-term studies with sublethal amounts of the water-soluble fraction of petrochemicals (the part that persists in the environment) can be conducted routinely under laboratory conditions.

SECTION 5

AN INTERLABORATORY CALIBRATION EXPERIMENT UTILIZING *Capitella capitata*

An interlaboratory calibration toxicity test involving three laboratories (California State University, Long Beach; U.S. EPA Environmental Research Laboratory, Narragansett, Rhode Island; and State Marine d'Endoume, Marseille) was planned and carried out with the assistance of Mrs. Carol Pesch of the Environmental Research Laboratory. Dr. Gerald Bellan and Dr. Denise Bellan-Santini were the participants from Marine d'Endoume. *Capitella capitata* was selected as the test organism, and cadmium ($CdCl_2$) was selected as the toxicant. This is the first interlaboratory calibration experiment that has been conducted with a marine organism. Since the paper that describes this experiment and results has been published elsewhere, only the reference to it will be given: Reish, D.J., C.E. Pesch, J.H. Gentile, G. Bellan and D. Bellan-Santini. 1978. Interlaboratory Calibration Experiments Using the Polychaetous Annelid *Capitella capitata*. Marine Environ. Res. 1:109-118.

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Table 1

List of Polychaetous Annelida Which Are or Were in Laboratory Culture

Species	Stage Cultured To	Number of Generations	Length of Life Cycle (In Days)	Manual of Procedures	Status of Culture 1979
Neanthes arenaceodentata	Life Cycle	50+	120	Appendix 1	Maintained
Capitella capitata	Life Cycle	50+	30	Appendix 2	Maintained
Ctenodrilus serratus	Life Cycle	50+	10-15	Appendix 3	Maintained
Ophryotrocha diadema	Life Cycle	50+	10-15	Appendix 4	Maintained
Ophryotrocha puerilis	Life Cycle	20+	20-25	Appendix 4	Maintained
Dinophilus sp.	Life Cycle	50+	5	Appendix 5	Maintained
Dexiospira brasiliensis	Life Cycle	3	30	Appendix 6	Maintained
Polydora ligni	Life Cycle	3	50	Appendix 7	Not in Culture
Boccardia proboscidea	Life Cycle	3	100	Appendix 8	Not in Culture
Cirriformia luxuriosa	Two Years	incomplete	probably two years	Appendix 8	Not in Culture
Cirriformia spirabrancha	Life Cycle	1	475	Appendix 8	Not in Culture
Halosydna brevisetosa	Life Cycle	1	270-300	Appendix 8	Not in Culture

Table 2 Toxicity of Heavy Metals to Polychaetous Annelids
(in mg/l)

Species	Cadmium		Chromium		Copper		Lead		Mercury		Zinc	
	96 hr LC50	28 day LC50	96 hr LC50	28 day LC50	96 hr LC50	28 day LC50	96 hr LC50	28 day LC50	96 hr LC50	28 day LC50	96 hr LC50	28 day LC50
Neanthes arenaceodentata												
Adults	12.0	4.0	1.0	0.55	0.3	0.25	10.0	3.2	0.22	0.17	1.8	1.4
Juveniles	12.5	3.0	1.0	0.7	0.3	0.14	7.5	2.5	0.1	0.09	0.9	0.9
Capitella capitata												
Adults	7.5	0.7	5.0	0.28	0.2	0.2	6.8	1.0	0.1	0.1	3.5	1.25
Trochophores	0.22	---	8.0	---	0.18	---	1.2	---	0.014	---	1.7	---
Ophryotrocha diadema	4.2	---	7.5	---	0.16	---	14.0	---	0.09	---	1.4	---
Dinophilus sp.	0.8	---	0.82	---	0.026	---	2.5	---	---	---	0.22	---
Halosydna johnsoni												
Adults	13.0	6.1	4.7	1.45	0.15	0.084	6.25	6.0	0.3	0.27	6.0	3.0
Trochophores	---	---	1.2	---	0.1	---	---	---	0.028	---	2.2	---
Cirriformia luxuriosa												
Adults	15.0	3.5	---	---	0.9	0.65	---	---	---	---	> 15.0	8.6
Juveniles	---	---	---	---	0.35	0.2	---	---	---	---	---	---
Cirriformia spirabrancha	---	---	---	---	0.75	0.2	---	---	---	---	---	---
Ctenodrilus serratus	4.3	---	4.3	---	0.33	---	7.2	---	0.04	---	7.1	---
Dexiospira brasiliensis	---	---	---	---	0.96	0.03	---	---	---	---	3.0	0.16

Table 3 The Effect of Heavy Metals on
Reproduction in Laboratory Reared
Polychaetous Annelids

Metal and Species	Highest Concentration at Which Reproduction Occurred	Concentration at Which Reduction in Significant Reproduction Occurred
Cadmium		
Ctenodrilus serratus	2.5 mg/l	2.5 mg/l
Ophryotrocha diadema	1.0	1.0
Chromium		
Neanthes arenaceodentata	0.05	0.0125
Capitella capitata	0.4	0.1
Ctenodrilus serratus	0.5	0.05
Ophryotrocha diadema	0.1	1.0
Copper		
Capitella capitata	0.1	0.05
Ctenodrilus serratus	0.1	0.1
Ophryotrocha diadema	0.1	0.25
Lead		
Ctenodrilus serratus	1.0	1.0
Ophryotrocha diadema	0.5	5.0
Mercury		
Ctenodrilus serratus	0.05	0.05
Ophryotrocha diadema	0.1	0.1
Zinc		
Capitella capitata	0.1	0.05
Ctenodrilus serratus	2.5	0.5
Ophryotrocha sp.	0.5	0.5
Dexiospira brasiliensis	0.05	0.01

Table 4 Toxicity of the Water Soluble Fraction of Two Oils on Polychaetous Annelids

Species	Fuel Oil #2		So. Louisiana Crude	
	96 hour LC50	14 day LC50	96 hour LC50	14 day LC50
Capitella capitata	no deaths at 100%	70%	no deaths in 100% oil	70% survival in 100% oil
Ophryotrocha diadema	67% in 30% oil	28	65	55
Cirriformia spirabrancha	no deaths at 100%	70	no deaths in 100% oil	50
Ctenodrilus serratus	47	38	96% survival in 100% oil	72% survival in 100% oil
Dexiospira brasiliensis	91	56	-	-

Table 5 The Effects of the Water Soluble Fractions of Two Oils on Reproduction in Two Species of Polychaetous Annelids

Oil	Highest Concentration With Reproduction at 21 days		Concentration at Which a Significant Difference in Reproduction Occurred	
	Ctenodrilus	*Ophryotrocha*	*Ctenodrilus*	*Ophryotrocha*
So. Louisiana Crude	75% WSF	75% WSF	50% WSF	50% WSF
Fuel Oil #2	25	10	25	10

APPENDIX A

CULTURE AND BIOASSAY PROCEDURES FOR POLYCHAETOUS ANNELIDS: *Neanthes arenaceodentata*

CULTURE TECHNIQUES

Equipment and Supplies

The equipment and supplies needed to maintain a continuous supply of *Neanthes arenaceodentata* at the rate of 1,000 to 2,000 per month are neither elaborate nor expensive. Approximately 20 m^2 of either shelf or table surface is needed to rear this quantity of worms. While it is not essential, a constant temperature laboratory set at 19±1°C, insures against the possible effects of either abnormally high (>25°C) or low (<17°C) temperatures. *N. arenaceodentata* is capable of withstanding temperature fluctuations of 19±4°C without any measurable stress. Natural, filtered seawater at normal salinity (35 °/oo) is a satisfactory source of seawater; however, success has been obtained using artifical sea salts. *N. arenaceodentata* can tolerate salinity variations of 35±5 °/oo without adverse affects. A running seawater system is not required; approximately 1,500 liters of seawater are needed per month to raise 1,000 to 2,000 specimens. A central compressed air system is the most convenient source of air, but several aquaria pumps could supply sufficient air to culture *N. arenaceodentata*. A dissecting microscope with light is required to examine specimens prior to use in an experiment. A hand lens and flashlight are helpful in examining the condition of the eggs and larvae. Glass containers required include 3.78 ℓ (1 gallon) jars, standard petri dishes (may be plastic) and 37.8 ℓ (10 gallon) to 56.7 ℓ (15 gallon) aquaria. Additional supplies needed include 12 cm diameter plastic covers for the glass jars, forceps, fine brushes, plastic tubing, air stones, an outside aquaria filter system, charcoal, and glass wool. Quantities of the dried green alga *Enteromorpha* sp. and commercially prepared alfalfa flour are needed for a food supply.

Life History

N. arenaceodentata is widely distributed throughout the world; it has been collected in Europe, New England, Florida, California, Baja California, and the Central Pacific. It is present within the sediment, especially in estuarine environments. It may be present in large numbers; as many as 1,000/m^2 have been observed. Specimens live in the benthos where they construct tubes consisting of sediment, food, and feces, which is held together by mucus.

Specimens measure up to about four cm in length and are tan to yellow-tan in color. Since many species of Family Nereidae appear alike under low magnification, *Neanthes* can be distinguished from the other species by the possession of a continuous ring of paragnaths around areas V - VII of the proboscis (Figure 1) and by a hooked tip present on the blade of the neuropodial heterogomph seta (Figure 2).

It is impossible to distinguish immature males from females on the basis of morphology. However, a behavioral difference occurs that can be utilized to distinguish sexes. Males will fight males and females will fight females. This fighting behavior consists of extending the jaws to grasp the opposing worm (Figure 3). They can be cannibalistic. A male and female placed together will come alongside each other and lie side by side. If one specimen has constructed a tube, the two will lie within the tube until eggs are laid. It is possible to detect this behavior difference in young specimens possessing 20-25 segments. In order to determine the sex of an immature worm, place it in a petri dish with a female containing developing eggs within her coelom and observe the behavior of the two when they come in contact with one another. If they come to lie side by side, then the immature worm is a male.

The eggs of *Neanthes* are formed within the walls of the parapodia. Shortly thereafter, they break free and mature within the coelom. The muscle cells, especially the longitudinal muscles, slough off and are digested by the eleocytes that transfer material to the maturing eggs. The mature eggs of *Neanthes* are large, measuring 500-600 μ in diameter. During maturation of the egg, about 75 percent of the body weight of the female is transferred to the eggs. The eggs are probably passed through bilateral breaks in the body wall between successive parapodial lobes. The fertilized eggs are clumped in the central part of the tube around the mid-body region of the male.

Development of the young proceeds within the tube until the 18-21 setigerous segment stage (Figures 4-6). The developing eggs are incubated by the male who circulates water with his body undulations. These undulations provide a continuous source of oxygenated water that is essential for development of the eggs. The fertilized eggs are yellow as the result of the large amount of yolk material, but as the yolk supply is utilized during development, the young become tan in color. *Neanthes* lacks the free-swimming trochophore larval stage characteristic of most polychaetes. The shape of the larval body is distorted as a result of the large quantities of yolk material; in fact, the yolk bodies may be observed in the future digestive tract of larvae up to nearly the 18 setigerous segment stage (Figure 4-6). The young worms leave the tube of the parent when they attain 18-21 setigerous segments (Figure 7). Shortly after leaving the parent's tube, they construct a tube and commence feeding. Under laboratory conditions, it takes three to four months for *Neanthes* to complete its life cycle at about 20-22°C. Presumably, a longer time is required in the field because of lower temperatures. Under laboratory conditions, the male is capable of reproducing at least a second time after the conclusion of the initial incubation period.

Techniques of Handling *Neanthes*

Adults--

Stock colonies of adults can be maintained at room temperature in aquaria of about 37.8 to 56.8 liter size with a maximum population concentration of approximately 75-100 worms per aquaria. The aquarium must be provided with at least two air stones and an outside filter system for aeration (Figure 8). Add about 1.5 g of alfalfa flour and/or *Enteromorpha* per 56.8 ℓ aquarium per week. The alfalfa flour should be mixed with seawater prior to use.

If all specimens are going to be used for experimental studies within a month, it will not be necessary to change the water in the aquarium; however, the water level should be checked periodically and double distilled water added to keep the level constant. The air stem in the outside filter system must be checked weekly and cleared of accumulated salts. If the specimens are not being utilized within a month, the water should be changed at about four to six weeks.

Large quantities of the green alga *Enteromorpha* sp. can be collected from estuaries during high tide. This genus is widespread in temperate estuarine environments of the world and frequently flourishes in the spring or fall months. The alga is hand-collected and washed at the site to remove as much adhering debris and sediments as possible. It is then spread out on chicken wire and allowed to air-dry. After drying, it can be stored in plastic bags for an indefinite period of time. In areas of high humidity it may be necessary to dry it in a warming oven (30-35°C). The dried *Enteromorpha* is soaked in seawater prior to refeeding the worms. The alga should be kneaded so that the individual branches of the alga separate from one another. The alga is then ready to feed to the worms.

Reproductive Specimens--

Females with maturing eggs in the coelom appear yellow-orange. Since sexual maturity will be reached about two months after the aquarium is established with juvenile worms, all specimens should be removed from the aquarium and females with eggs should be set aside in a petri dish. Remove a large worm with a fine paint brush from the aquarium, and place it in the dish with the female. Determine the sex of the second worm by the fighting response. If the unknown worm is a male, establish a 3.78 ℓ jar with the couple, and feed them *Enteromorpha*. Examine them two to three times a week until the eggs are laid. Note when the eggs were laid and continue to feed the worms on schedule. Examine the developing embryos every other day beginning at two weeks for emergence from the parent's tube. Since the male can become cannibalistic after the emergence of his offspring, it is important to remove the parent. The male may be utilized a second time or discarded. Do not use it as an experimental animal.

Larvae--

No special care is required for specimens having less than 18-21 setigerous segments. These nonfeeding larvae are cared for by the male parent within his tube.

Immature Worms--

After the young worms leave the tube of the parent, they should be removed as soon as possible to prevent being eaten by the parent. Place 50 to 75 young worms in a 56.8 ℓ aquarium with about 50 ℓ of seawater. Feed them 1.5 g of alfalfa flour initially and once weekly. Cut down the amount of food fed if the majority of the food has not been eaten.

Problems in Culturing--

Most of the difficulties in culturing *Neanthes* can be attributed to the lack of regular, systematic care. Since there may be a large number of various sized containers such as petri dishes, 3.78 ℓ jar aquaria, and 17.8 to 56.8 ℓ aquaria, it is recommended that each container be numbered and records kept. It is possible to minimize some of the routine record keeping by keeping basic data written on the outside of the container. Arranging the containers by stages will facilitate routine observations and feeding. It is recommended that all routine culture care be done on a specific day each week.

Feeding problems--The two primary feeding problems are insufficient soaking and kneading of the algae and overfeeding. Both problems lead to the appearance of a white fungal growth over the surface of the algae that the worms cannot penetrate to feed. As a general rule, it is better to underfeed than to overfeed. The individual filaments of the soaked and kneaded *Enteromorpha* should separate from one another when placed in an aquarium. If all the food is in one clump, the worm will not feed.

Enemies--Fungal growth can cause the worm to abandon its tube and build a second one elsewhere. Microorganisms, such as protozoa and copepods, may cause problems by eating the food or attacking the worm, but this has not been proven as yet. Amphipods may feed upon developing embryos since larvae disappear from the parent's tube whenever these crustaceans become established in the aquarium.

Cannibalism--As discussed above, like sexes fight, and it is possible for one to eat the other. Like sexes should never be placed together within a petri dish. Also, do not overcrowd worms in aquaria.

Abandonment of fertilized eggs by male--About 10 to 15 percent of the time the male may abandon his tube of developing eggs. Eggs left in a tube abandoned by a male will not survive. This results in the death of the zygotes, and attempts to provide aeration for these abandoned eggs have been successful only with a magnetic stirring device (Hinegardner, 1969). The cause of this abandonment is unknown.

Parent male eating developing eggs or larvae--About 10 to 15 percent of the time male parent will eat his offspring, especially during the late stages of development. The amount of food present does not seem to alter this behavior. The cause of this behavior is unknown. Since upwards to one-third of the fertilizations may result in either abandonment or cannibalism of the offspring, the only apparent solution of this problem is to allow for this amount of loss in culturing this species.

TOXICITY TEST

Equipment and Supplies

In addition to the equipment and supplies listed above, 500 ml Erlenmeyer flasks or other suitable containers, for example a Carolina dish, culture dishes, etc. and white enamel pans are needed.

96-Hour Experiments with Juveniles or Adults

Remove clumps of Neanthes from the stock colony, and place them in a white pan filled with enough seawater to reach a depth of one cm. Allow the worms to free themselves from their tube masses if possible so as to minimize the amount of handling. Touching one end of the tube mass with a brush may hasten the worm's exit. Transfer the worm to a petri dish containing seawater. Several worms can be placed within the same dish if they are not left together for more than one hour. Separate out any female with developing eggs; the female will appear orange in color and after some practice all but the ones with ova in the early stages of development can be recognized without the use of a microscope. Do not use any females with developing eggs as test specimens. Females with developing ova can be utilized for reproductive stock. Examine all worms under a dissecting microscope for presence of ova or injuries. Discard all injured specimens. Place a single worm in a 500 ml Erlenmeyer flask containing 100 ml seawater and toxicant. Close the flask with a rubber stopper.

Use 20 specimens per concentration and a minimum of five concentrations of toxicant plus control. Examine each worm daily for death. Discard all specimens at the end of the test.

Death in *Neanthes*--

Death in *Neanthes* is defined as the absence of movement when the flask is gently rolled or when a worm is gently poked. A dead *Neanthes* typically appears white; its proboscis is everted exposing its jaws, and it has abandoned its mucoid tube.

Many behavioral changes in *Neanthes* can be observed that will assist the experimenter in determining the health of the worm. If the concentration is highly toxic, the worm will react violently by everting its proboscis and moving its body in twisting motions. If this activity begins when the worm is initially placed with the toxicant, then death will follow within an hour or less. A similar behavioral response is elicited if *Neanthes* is placed directly into fresh water. If the twisting activity begins several hours or more later, the movement will not be as violent and death may take days.

Tube building also is an indication of the well-being of a worm. A healthy *Neanthes* will construct a mucoid tube on the bottom or along the side of the container. Slightly stressed worms will construct the mucoid tube along the side of the container at the air-water interface. With additional stress or effect of a toxicant the worm will fail to construct a mucoid tube.

Long-Term Experiment with Adults (28 days)

Separate out and examine the worms to be used in the experiment as outlined above. For a 28-day toxicity test the solution should be renewed a minimum of every four days. It will be necessary to feed *Neanthes* because of the length of the test. Soak dried *Enteromorpha* sp. in a petri dish containing seawater. Knead the alga so that it is well soaked. Use about 0.1 g of dried *Enteromorpha* sp. (0.2 g wet weight) per worm. The *Enteromorpha* sp. should be soaked in the same concentration of seawater plus toxicant in which it is going to be used. Place the appropriate amount of kneaded *Enteromorpha* sp. for one *Neanthes* in a petri dish at the specific concentration of toxicant where the worm will be tested. Place a single *Neanthes* in the center of algal mass. Continue this procedure until all experimental worms have been so separated. Transfer algae and worm to experimental flask, and commence the experiment. Examine daily for the first 96 hours, then at 7, 14, 21, and 28 days for death. (This experiment can serve also as a 96-hour experiment.) Discard all specimens at the conclusion of the experiment.

Death--

The description of death in *Neanthes* given under the 96-hour toxicity test applies to this long-term experiment with some additional behavioral changes as the result of the introduction of *Enteromorpha* sp. as food. If *Neanthes* is able to construct a tube in a moderately toxic solution it will build one along the sides of the container at the air-water interface. It may or may not incorporate the alga in its tube; if it does, it may or may not feed upon the alga. The amount eaten per unit time may be less than observed for specimens in the control. *Neanthes* in control solutions or nontoxic solutions will construct mucoid tubes in the alga on the bottom of the container. Fecal pellets will be noted within a 24-hour period.

Long-Term Experiments to Study the Effects of a Toxicant on Reproduction, Egg Production, and F_1 Larval Survival--

Place *Neanthes* tube masses in white enamel pans and separate out specimens weighing about 10-15 mg wet weight, (35-45 setigerous segments). The worms have been out of the parental tube for seven to ten days. Place four worms in a petri dish; examine all specimens under the dissecting microscope; and discard all injured ones. Place the four worms in a 3.78 ℓ glass jar containing 2,500 ml of toxicant. It may be necessary to use a brush to free the worms from the petri dish. Add 0.3-0.4 g wet weight (0.2 g dry weight) of kneaded *Enteromorpha* sp. to each jar. Aerate with an air stone. It is convenient to use about 15 aquaria (60 worms) per concentration to take into account any unusual sex ratio or death of worms at the higher concentrations. Add food once a week. For the second and third feeding, add about 0.45 g wet weight (0.3 g dry weight). For feedings beyond the third week, add 0.6 g wet weight (0.4 g dry weight). Do not add any food if the worms have failed to eat during the previous week. For the best results in long-term static bioassays, the water should be renewed every three weeks.

If the purpose of the experiment is to study the effect of the toxicant on egg production, then examine each female for the presence of developing ova within her coelom. All jars containing females with developing ova should be marked to facilitate examination every two to three days to see whether or not the eggs have been laid. When the eggs have been laid, carefully remove the tube mass containing the eggs to a petri dish, and count under a dissecting microscope the number of eggs present. Discard the eggs after counting. Maintain the jar if another male and female are present; if not, either transfer the specimens to another jar within the same concentration series or destroy.

If the purpose of the experiment is to study the effect of the toxicant on the F_1 or F_2 generation, follow the procedure outlined above through the egg laying stage, but do not remove the tube mass containing the eggs. Instead, remove all other tube masses from the jar with a pair of long forceps. Record the date of egg-laying and begin observing the jar on a daily basis after 15 days for emergence of juvenile worms from the parent's tube mass. Such juveniles should possess a dark line extending throughout much of their length. This is food visible in the gut and indicates that the worm has commenced feeding. These juveniles can be removed from the jar, counted, and either discarded or used to establish a new jar according to the procedures outlined above, if the study is planned to extend through the F_2 generation.

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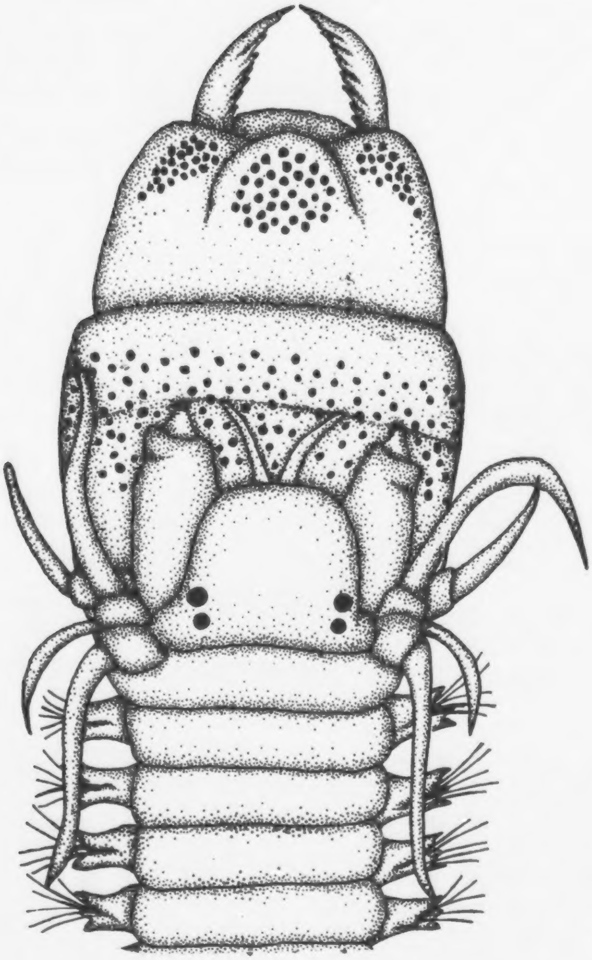

Figure 1. *Neanthes arenaceodentata*, anterior end, with everted proboscis.

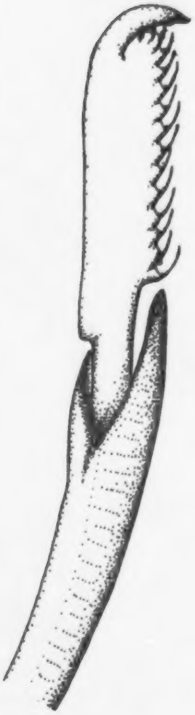

Figure 2. *N. arenaceodentata*, neuropodial heterogomph falcigerous seta with hook at tip of the blade.

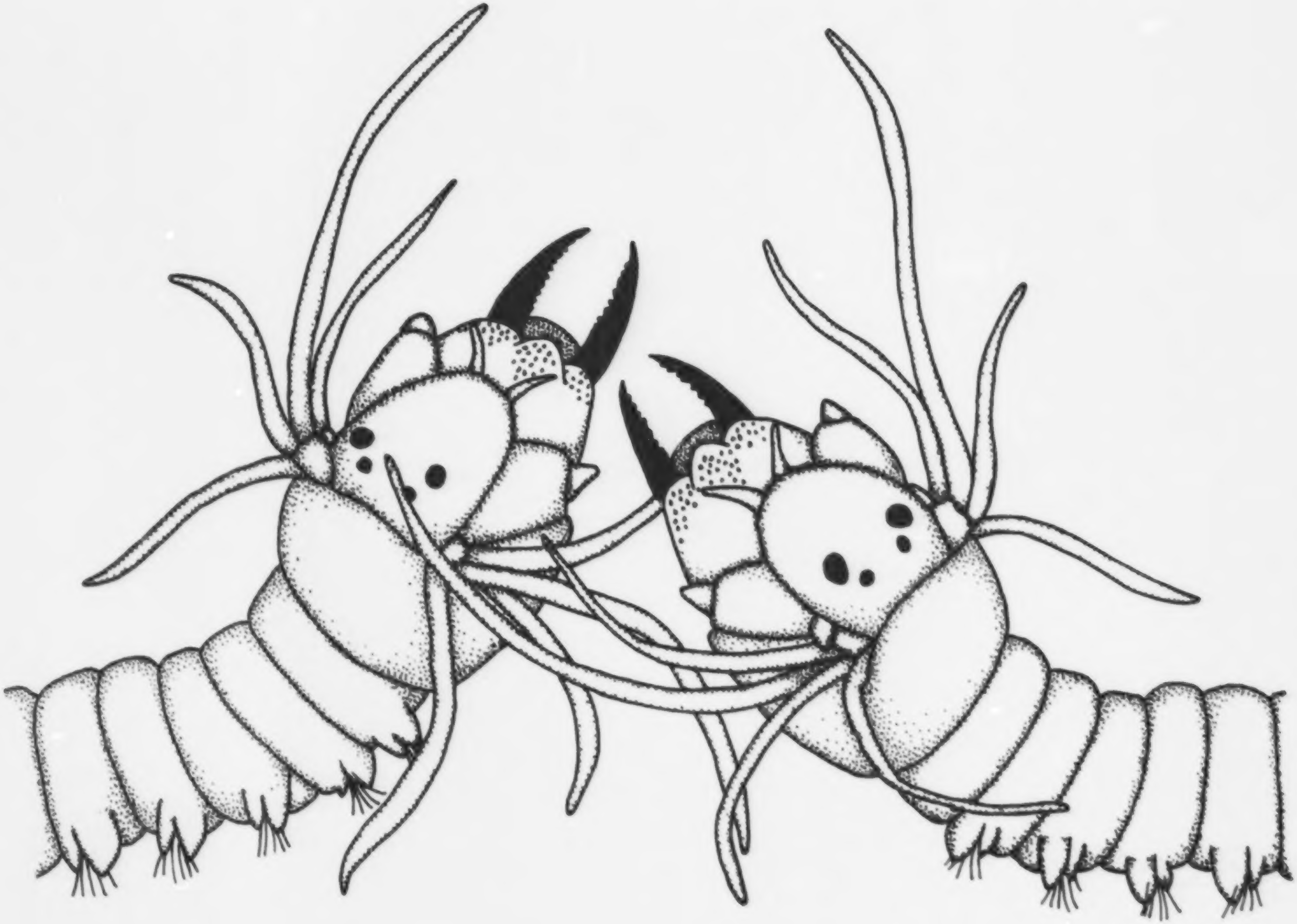

Figure 3. *N. arenaceodentata* of the same sex in fighting position.

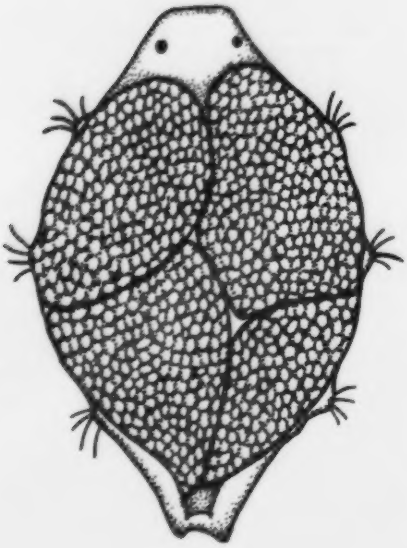

Figure 4. *N. arenaceodentata*, three segmented stage.

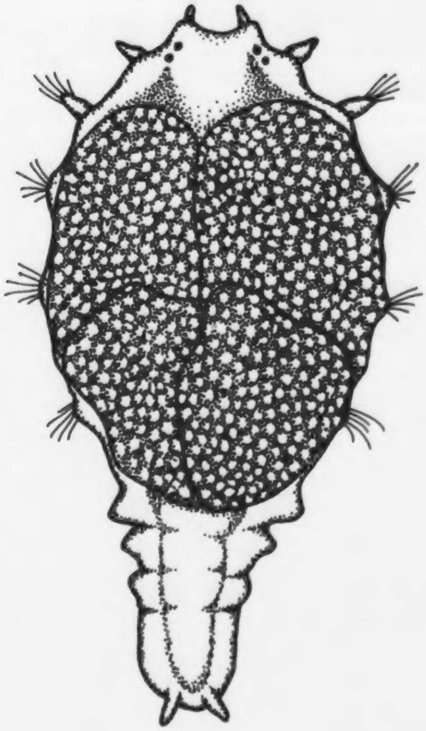

Figure 5. *N. arenaceodentata*, four segmented stage.

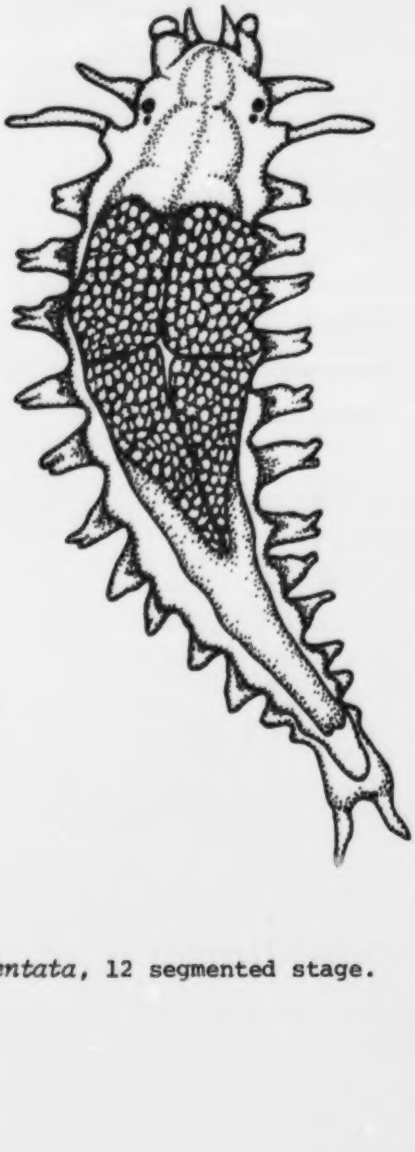

Figure 6. *N. arenaceodentata*, 12 segmented stage.

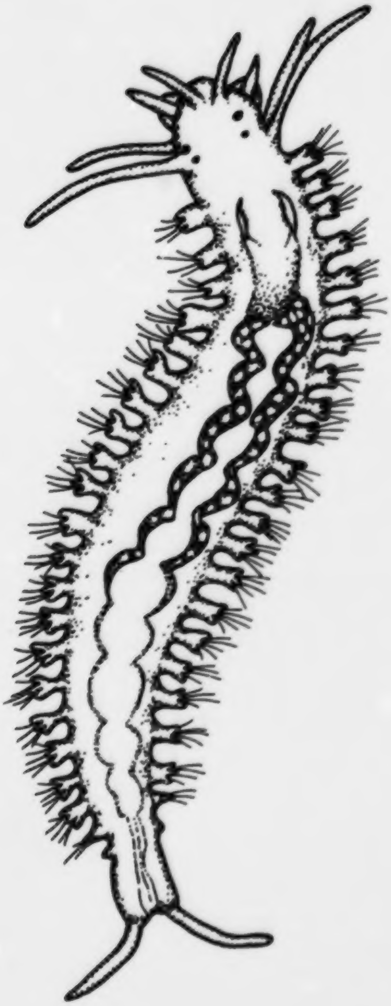

Figure 7. *N. arenaceodentata*, juvenile, with 21 segments, which has left the parent's tube and commenced feeding.

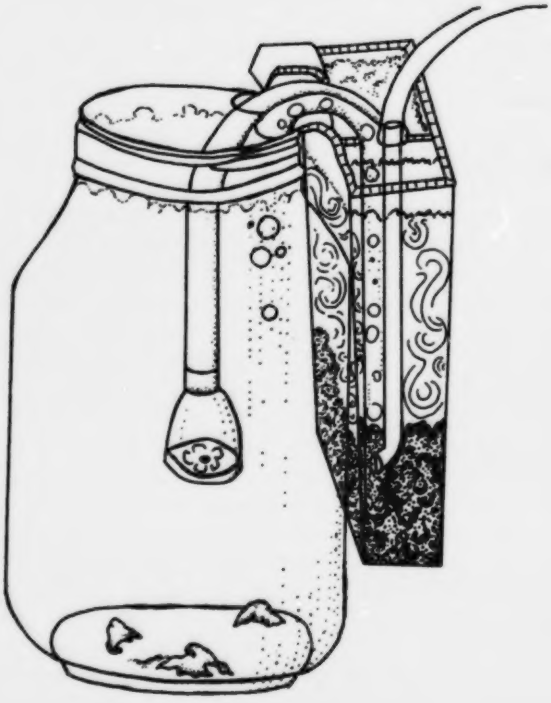

Figure 8. One gallon (3.78 ℓ) aquarium system fitted with an outside filter system used to culture *Neanthes sp.* adults, especially those specimens approaching sexual maturity.

APPENDIX B

CULTURE AND BIOASSAY PROCEDURES FOR POLYCHAETOUS ANNELID: *Capitella capitata*

CULTURE TECHNIQUES

Equipment and Supplies

The equipment and supplies required to maintain a population of 3,000-5,000 specimens of *Capitella* are neither elaborate nor expensive. Approximately three to four m^2 of shelf space is needed to culture and maintain this size population of *Capitella*. For best results, it is advantageous, although not essential, to culture *Capitella* in a constant temperature laboratory maintained at 19±1°C. Elevated temperature, especially above 23-24°C may cause fouling of the food, which leads to the death of the specimens within the colony. Growth rate below about 17°C is markedly reduced leading to an increase in length of time required for completion of its life cycle. Natural, filtered seawater at normal salinity (35 $^o/oo$) has been the most satisfactory media to culture *Capitella*; however, it is capable of withstanding lower salinities (25 $^o/oo$). A running seawater system is not required; approximately 100-150 liters of seawater are needed per month to maintain a population of 3,000-5,000 specimens. A piped compressed air system fitted with many outlets is the most convenient source of air for *Capitella*; however, a few aquaria pumps could supply sufficient air. A dissecting microscope with a light source is required for examination of specimens prior to use in experiments. Glass containers required include 3.78 ℓ jars and standard sized glass or plastic petri dishes. Additional supplies include 12 cm diameter plastic covers for the jars, jeweler's forceps, fine brushes, plastic tubing, and air stones. Dried quantities of the green alga *Enteromorpha* sp. and dried fish food flakes are needed as a food supply.

Life History

Capitella is a cosmopolitan species that is generally found in estuarine waters. It has been described as a non-competitive or opportunistic species because it flourishes in the absence of other polychaete species (Barnard, 1970). *Capitella* is found in large numbers in the vicinity of domestic outfall sewers, and it has been used as an indicator of such altered environments (Pearsons and Rosenberg, 1978). Grassle and Grassle (1976) have described six sibling species of *Capitella* that are distinguished by the morphology of the anterior and posterior ends, the arrangement of teeth on the hooded hooks, the weight of the specimens, method of reproduction, the diameter of eggs, the number of eggs laid, and the length of larval life. Using the sibling species classification of Grassle and Grassle, the present population of *Capitella* belongs to Type I.

Specimens generally measure less than two cm in length although larger individuals occur especially in the vicinity of domestic outfalls. *Capitella* is one of the few species of polychaetes that exhibit sexual dimorphism. Specialized genital hooks appear on the mid-dorsal region of setigerous segments eight and nine in males (Figure 9). Females lack these setae (Figure 10). Young adults may be sexed easily by observing the anterior region under the dissecting microscope and checking for the presence or absence of these genital hooks.

Copulation occurs, but it is rarely observed. Sperm are transferred to the female with fertilization occurring either internally or at the time of discharge of the eggs. The tube of *Capitella* is a loosely constructed structure that consists of fecal material, substrate, and potential food. The tube is open at either end, and the specimen is capable of building another one if the initial tube is abandoned. The female places the fertilized eggs around the inner surface of her tube (Figure 11). The eggs remain fixed in this position, presumably by a mucoid secretion, until the trochophore stage. There is a considerable amount of variation in the number of eggs laid, but under laboratory conditions, it usually ranges from 200 to 400.

The female incubates the fertilized eggs during the early developmental stages. Incubation consists of periodic body undulations of the female that circulate water through the tube. These undulations are apparently essential for development because attempts to provide aeration to developing zygotes following female abandonment have been unsuccessful. The fertilized eggs are initially white and measure about 250 μ in diameter. As development continues, the zygotes become darker, and at the trochophore stage they appear grey-green.

The trochophore stage is reached about four to six days after egg-laying (Figure 12). Trochophores are capable of moving freely within the tube either by ciliary movement or by contraction of longitudinal muscles. The trochophore may either swim free of the tube and become planktonic or proceed directly into the metatrochophore stage (Figure 13) and begin to form its own tube as a side branch from the parent tube. The planktonic trochophore, if it occurs, is of short duration, and it soon settles to the substrate and develops into the metatrochophore stage.

The trochophore and metatrochophore stages last one to two days before resembling a juvenile adult (Figure 14). Growth is rapid and temperature dependent. Eggs begin to develop in the coelom of the maturing female in about 20 days, and fertilized eggs are laid at 25-40 days at 20°C.

Both sexes are capable of reproducing more than once. Females have been observed to have had three successful egg layings under laboratory conditions. The second egg laying occurs about five to ten days after the first brood has left the tube. Under laboratory conditions, both sexes appear to lose their vigor with increasing age as indicated by body color. The characteristic internal blood red color of young adults becomes dull red in color with increasing age.

Techniques of Handling *Capitella*

Adults--

Stock colonies of adults can be maintained at 20°C in 3.78 ℓ (1 gal) jars (Figure 15). Add 2,500 ml filtered seawater to each jar. Provide areation with an air stone connected to a plastic tube and air supply. Adjust the pressure in the system to a low level. The top of the jar can be covered with a glass or plastic top to minimize evaporation. Many adults (10-30) can be placed in a single jar since *Capitella* does not exhibit cannibalistic behavior. Add about 0.15 g dried *Enteromorpha* sp. per week to each jar. Commercial fish flakes, Tetramin, or Biorell are useful food; however, prior to use these flakes should be ground into a fine powder. Add about 0.1 g of this fine powder per jar per week.

If specimens are not being removed from a particular jar for experimentation, the contents of one jar should be divided into two or three additional jars about every three weeks. Decant most of the seawater from the jar until about 100 ml remains. Pour the remaining water and worm tube masses into a few petri dishes and allow them to go undisturbed for several minutes. *Capitella* will usually crawl free from its mucoid tube. Remove the specimen with a fine brush and place into a new dish with seawater. Water should be changed and food added at this time. If specimens are being removed from a particular jar for experimentation, it will not be necessary to divide the population; however, the water should be changed about every three weeks. The colony can be reduced in size, but still maintained at a reduced expenditure of time, by placing some of the jars in a cold bath maintained at 15-17°C. Add food to each jar about twice a month. A colony can be maintained for about two months under these conditions without examination or changing the water. The colony can be built up by returning the jars to 20°C and following the above culture procedures.

Trochophore Larvae--

Many of the techniques used to handle trochophores (Figure 12) and metatrochophores were outlined under the adult culture section above. No special techniques are required for handling the larvae in a mixed population. Trochophores can be freed from their parent's tube under the dissecting microscope and 25 pipetted (with a Pastuer pipette) into a separate jar. The initial food should consist of about 0.1 g of finely ground *Enteromorpha* sp. or powdered Tetramin.

Metatrochophore Larvae--

The metatrochophore larvae in Type I of *Capitella* is short-lived and does not require any special techniques (Figure 13).

Food Preparation--

The green alga, *Enteromorpha* sp. is collected from estuaries during high tide. This genus is widespread in the temperate estuarine environments of the world and frequently flourishes in the spring or fall. The alga is hand-collected and washed at the site to remove as much adhering debris and sediments as possible. Then it is spread out on chicken wire and allowed to air-dry. When it is dry, it is collected and stored in plastic bags for an indefinite period of time. In areas of high humidity it may be necessary to dry it in a warming oven (30-35°C). Prior to feeding, *Enteromorpha* sp. should be soaked in seawater and kneaded.

Enteromorpha sp. powder is prepared by grinding up the alga in a mortar and pestle until fine. The powder may be added directly to the jar or mixed with seawater prior to feeding. Tetramin is ground into a powder in the same way as *Enteromorpha* sp.

Problems in Culturing--

Most of the difficulties and failures in culturing *Capitella* can be attributed to the lack of regular, systematic care. Since there may be a large number of jars involved, it is recommended that the basic data be written on the outside of the jar. Arranging the jars by stages facilitates the routine observations and feedings. It is recommended that all routine culture care be done on a specific day each week.

Feeding problems--The two primary feeding problems are feeding too much food or not soaking or kneading the alga sufficiently prior to use. Both situations lead to appearance of a white fungal growth over the surface of the alga that the worms cannot penetrate to reach the food. The individual filaments of the soaked and kneaded *Enteromorpha* sp. should be able to separate from one another when placing it in the jar. White fungal growth can also occur when too much Tetramin powder is added. Each jar should be examined prior to feeding to determine whether or not additional food is required.

Enemies--Fungal growth can result from overfeeding, improper conditioning of food, or insufficient dissolved oxygen. All these causes of fungal growth can be prevented by proper and periodic care. Microorganisms, especially ciliated protozoans, nematodes, and copepods, are almost always present in the stock colonies, and they are almost impossible to eliminate. Whether or not they feed upon *Capitella* is unknown, but they compete for food. If the population of these organisms becomes large, they can be minimized by removing the clumps of worms from the aquarium just prior to feeding and placing them in a new container with filtered seawater.

Abandonment of Fertilized Eggs by Female--Occasionally the female may leave her tube of fertilized eggs. Attempts to provide aeration for these abandoned eggs have been unsuccessful. The only known cause of this condition is either rough handling or too much handling when examining the worm under the dissecting microscope. Abandonment of the tube also occurs within the stock colony; the causes of this problem are unknown, and no solution is known. Fortunately, this situation does not occur frequently enough to warrant searching for a solution.

TOXICITY TEST PROCEDURES

Equipment and Supplies

In addition to the equipment and supplies listed above, the following supplies are needed to conduct toxicity tests with *Capitella*: 500 ml Erlenmeyer flasks (or Carolina dishes, petri dishes, etc.), stender dishes, and white enamel pans.

Collection and Examination of Experimental Animals

Remove clumps of *Capitella* from stock colonies, and place them in white enamel pans with sufficient seawater to reach a depth of one cm. Since *Capitella* lives within a mucoid tube, it is convenient to allow them to crawl free of their tubes. If a large number of specimens is required it is advantageous to have several pans containing tube masses of *Capitella*. As the worm frees itself from the tube, remove it with a fine brush, and place it in a petri dish. Proceed to the next pan, and continue to examine each pan periodically until enough specimens have been obtained. Examine each specimen under the dissecting microscope, and remove any female containing developing ova within her coelom. Developing ova appear as bilateral, segmentally arranged masses of white tissue ventrally located beginning about segment 12. These females can be utilized for establishing reproductive colonies. Remove and destroy any injured specimens.

If trochophore larvae are to be used as experimental organisms, examine clumps of *Capitella* under the dissecting microscope, and look for females incubating embryos (Figure 11). If the embryos appear white, set the female and her eggs aside for establishing a separate stock colony. If the embryos appear grey-green, place the female and her young in a separate stender dish. Carefully tease open the tube and allow the trochophore to swim free of the tube. Remove the female and the tube. If 200 larvae or more are required, it will be necessary to obtain larvae from more than one female. If so, pipette all larvae into a common container, then distribute the larvae to the experimental chambers.

96-Hour Experiments with Juvenile or Adult *Capitella*

Use either petri dishes or 500 ml Erlenmeyer flasks as experimental containers. Place a single worm in a petri dish containing 25 ml of toxicant or an Erlenmeyer flask containing 100 ml of toxicant. Cover the container. Use 20 specimens per concentration and a minimum of five concentrations of toxicant plus control. Examine each specimen daily for death, and discard all specimens after 96 hours.

Death in *Capitella*--Dead *Capitella* may be difficult to distinguish from moribund ones. Death is defined as a lack of movement in response to gentle poking. Dying *Capitella* may fragment into two pieces. In this instance use the anterior end to determine death. A dead *Capitella* generally appears whiter than a living one and also slightly enlarged. It is usually lying on the bottom of the container free from any mucoid tube.

Long-Term Experiments with Juveniles (28 days)

Since the life history is short in *Capitella*, it is necessary to use younger worms for conducting a 28-day experiment. Separate out the appropriate aged specimens from white enamal pans. Examine each specimen under the dissecting microscope for injuries or females with developing egg masses. Place the selected specimens in containers as outlined for the 96-hour toxicity test. It will be necessary to feed the specimens but only at the initiation of the experiment. Soak dried *Enteromorpha* in a petri dish containing seawater. Knead the algae so that it is well soaked. Use about 0.1 g of dried *Enteromorpha* sp. (0.2 wet weight) per worm. The *Enteromorpha* should be soaked in the same concentration of seawater plus toxicant in which it is going to be used. Place the appropriate amount of kneaded *Enteromorpha* sp. for one worm in a petri dish or 500 ml Erlenmeyer flask with 25 ml or 100 ml of toxicant, respectively. Examine for deaths daily. The solution should be renewed every four days. Sublethal behavioral modifications in *Capitella* include the inability to feed or construct a mucoid tube. A healthy *Capitella* typically builds its tube within the *Enteromorpha* sp. on the bottom of the conainer; a stressed *Capitella* will construct its tube, if indeed it does, along the side of the dish at the air-water interface. Discard all specimens at the completion of the experiment.

96-Hour Experiments with Trochophore Larvae

Place ten trochophore larvae in each stender dish filled with 15 ml of the appropriate toxicant. Use ten dishes per concentration and a minimum of five concentrations plus control. Add 1.0 ml of a ground *Enteromorpha* sp.-seawater mixture of 1:100 by weight to each stender dish. The *Enteromorpha* sp. is required as food for the larvae and also to facilitate settlement and metamorphosis. Examine each dish daily and record the number of living specimens present. Frequently, a dead trochophore will have disintegrated within the 96-hour period and therefore cannot be seen.

Long-Term Experiments through F_1 Generation

Since this species possesses a short life history and abnormal larvae (Figure 16) can be induced in the presence of a toxicant, *Capitella* is a convenient species with which to conduct a whole life cycle toxicity test. The procedures outline a static test; if periodic water changes are to be made, care must be exercised not to disturb females incubating eggs in their tubes.

Separate out trochophore larvae as outlined above. A total of 100 larvae are needed per concentration. Pipette 25 larvae into a 3.78 ℓ jar that contains 2,500 ml of seawater and toxicant. Add 0.1 g ground *Enteromorpha* to the jar and feed once a week making certain that most of the food had been eaten the previous week. Do not feed if most of the food is present. Use four jars per concentration and a minimum of five concentrations plus controls. The 96-hour and 28-day toxicity tests should be conducted prior to this reproductive test to determine the toxicant concentrations. Examine the worms after 15 days for females with incubating embryos and every two to three days thereafter until the conclusion of the experiment (about 40 days). To examine the reproductive state of the worms, decant most of the water into another glass container, remove all the material from the bottom of the jar and place it into one or more petri dishes, and examine this material under the dissecting microscope for presence of females with developing embryos in their tubes. Remove these females and young and place in a separate dish and count the eggs. Replace all males and non-reproducing females in the jar with the appropriate toxicant concentration.

The data obtained from such an experiment furnish information to relate the concentration of the toxicant to survival, development of egg masses, reproduction, number of embryo produced, and the number of abnormal larvae produced.

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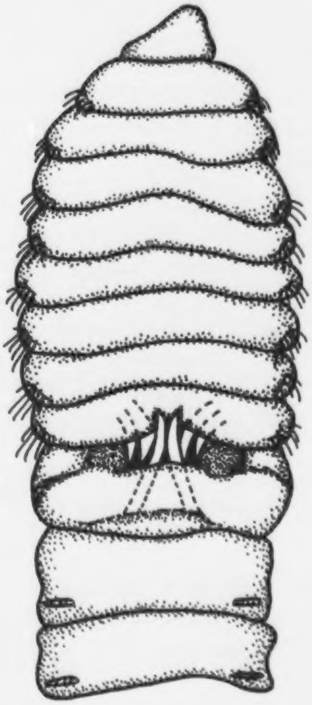

Figure 9. *Capitella capitata*, male, dorsal view of anterior end. Note the genital hooks in setigerous segments 8 and 9.

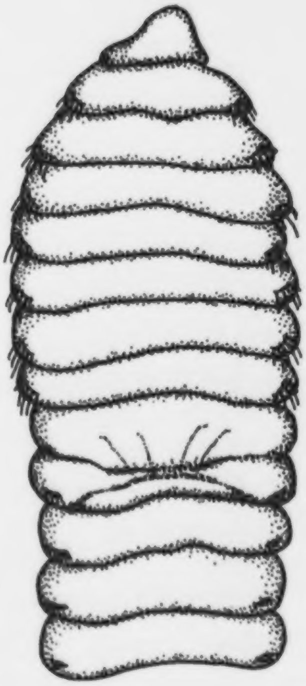

Figure 10. *C. capitata*, female, dorsal view of anterior end.

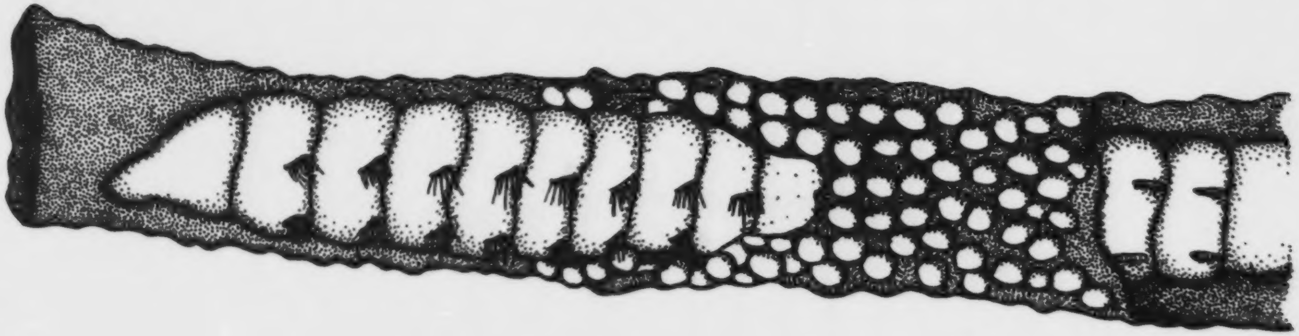

Figure 11. *C. capitata*, female, incubating eggs, lateral view. Note the mucoid tube with the fertilized eggs lining the inside of the tube.

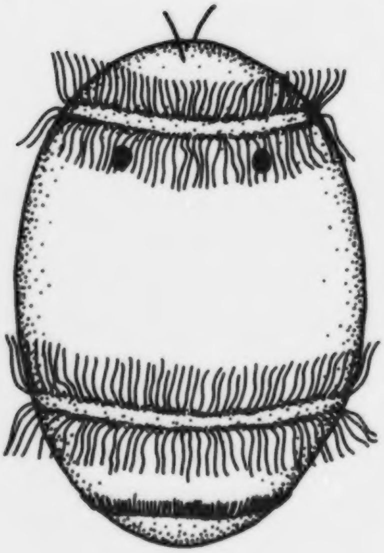

Figure 12. *C. capitata*, trochophore stage.

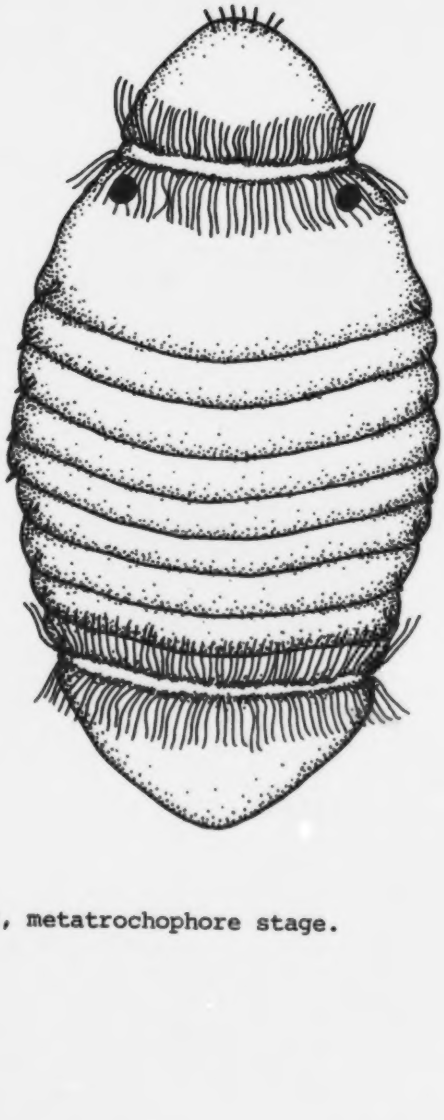

Figure 13. *C. capitata*, metatrochophore stage.

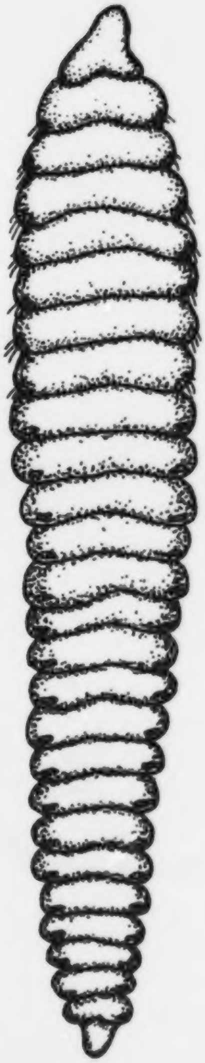

Figure 14. *C. capitata*, juvenile.

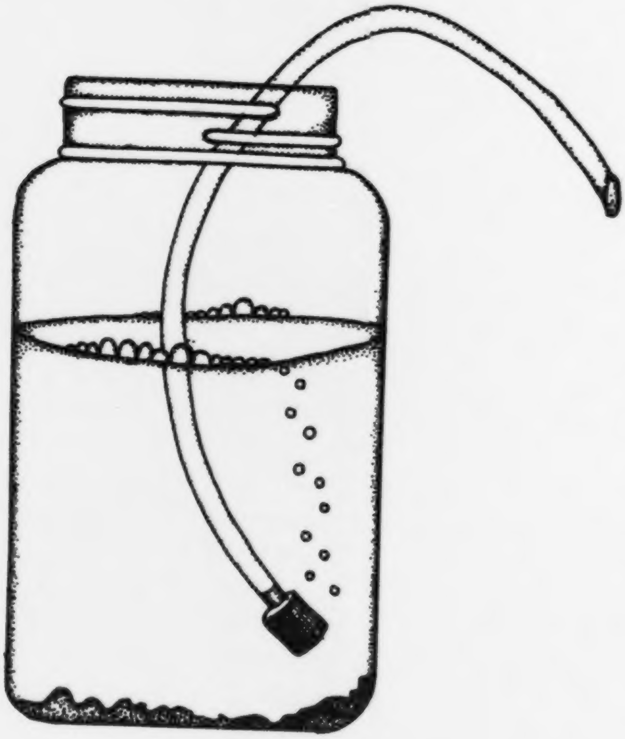

Figure 15. Gallon (3.78 ℓ) jar aquarium system for rearing *C. capitata*. The colony is located within the mass of food at the bottom of the jar. Air is supplied by an aquarium stone connected to an air compressor.

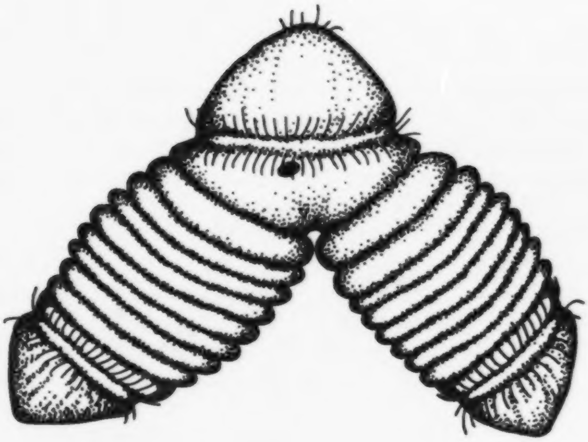

Figure 16. *C. capitata*, bifurcated abnormal larva induced by sublethal amounts of a heavy metal.

APPENDIX C

CULTURE AND BIOASSAY PROCEDURES FOR POLYCHAETOUS ANNELID: *Ctenodrilus serratus*

CULTURE TECHNIQUES

Equipment and Supplies

Since *Ctenodrilus serratus* is a minute polychaete, the equipment, supplies, and space required are minimal. Approximately one m^2 of shelf or table surface is required to rear 3,000 to 5,000 or more specimens per month. A constant temperature room set at 19±1°C, provides an ideal environment to culture this worm; however, *Ctenodrilus* can reproduce at temperatures as high as 25-26°C or as low as 15°C. Because of the small size of this worm, it is advantageous to use filtered seawater to minimize the introduction of microorganisms that may compete for food. The tolerance of *Ctenodrilus* to reduced salinities is unknown; this population has been cultured in normal salinity seawater (35 $^o/oo$). A static system is used to culture this species. Less than 100 liters of seawater are required per month to yield a monthly population of 5,000 specimens. A central compressed air system is the most convenient source of air for *Ctenodrilus*, although a population of this size could be supplied with one air pump. *Ctenodrilus* are cultured in 3.78 ℓ (1 gal) jars. Additional supplies needed include 12 cm diameter plastic or glass covers for the jars, forceps, fine brushes, petri dishes, pasteur pipettes, plastic tubing, air stones, graded screens, and a blender. Equipment includes a drying oven and dissecting and compound microscopes. Dried green alga, *Enteromorpha*, is used as food.

Collecting Techniques and Life History

This species is a minute polychaete that is widespread throughout the temperate region of the world. It is difficult to observe in the field because of its small size. The most convenient way to collect *Ctenodrilus* is to bring clumps of fouling organisms from boat floats or pilings into the laboratory. Examine a mussel under the dissecting microscope, and look for a small dark purple worm (Figure 17). This species lacks parapodia and moves over the surface in an earthworm-like fashion. Remove the worm with a pasteur pipette, and place it in a petri dish containing seawater.

Place a worm on a slide with a cover slip and examine under a compound microscope. The most conspicuous feature of the worm is its characteristic seta. The seta (Figure 18) is simple, and each is distally expanded and provided with four to six serrations along one side. The number of segments varies, but generally there are about 6-11 setigerous segments. Number of seta is usually three to four per side of a segment. It has been reported that *Ctenodrilus* reproduces in a variety of ways: transverse fission, protandric hermaphrodism, and internal gestation. A sexual transverse fission is the most common method of reproduction. Prior to reproduction by transverse fission, the dorso-anterior margin of each segment bearing setae becomes enlarged (Figure 19). This becomes the future prostomium. Growth of the future prostomium continues, and eventually each segment separates to form a new individual. A complete life cycle takes approximately 14-21 days.

Techniques of Handling *Ctenodrilus*

Since this species has such a short life history, it is unnecessary to use special techniques with the different stages. Stock colonies can be maintained at 19°C with a minimum of time spent. Place several worms in a petri dish with some food. *Enteromorpha* sp., a green macroalga, has been found to be the best food to use to culture this species. The *Enteromorpha* sp. is collected from estuarine areas, washed repeatedly in seawater, and then allowed to air-dry. *Enteromorpha* can then be stored indefinitely in plastic bags. The food is prepared by drying *Enteromorpha* sp. in an oven at 35°C for about 15 minutes. This crisp *Enteromorpha* sp. is then placed in a blender and chopped into fine powder. The powder is shaken through a series of fine screens to a size of less than 0.06 mm. This powdered food can also be stored for an indefinite period. In addition to petri dishes, several worms can be cultured in a single 3.78 ℓ jar. If several jars are being maintained, the *Enteromorpha* sp. powder can be weighed out and mixed with seawater at the amount of 350 mg per 25 ml of seawater. Each jar is fed at the rate of above one pasteur pipette drop of this mixture per worm per two weeks. Make a fresh mixture for each feeding. The specimens can be maintained in a jar for about one month. If each 3.78 ℓ is established with 25-50 worms, a potential yield of 500 to 1,500 specimens may be realized from each jar per month.

Problems in Culturing--

No special problems have been encountered in culturing *Ctenodrilus*. It is important to establish new stock jars on a monthly basis to minimize the growth of bacteria, protozoans, and copepods. By the use of microporous-filtered seawater, the buildup of microorganisms is minimized. There are no known enemies of *Ctenodrilus*.

TOXICITY TEST PROCEDURES

Equipment and Supplies

In addition to the equipment and supplies listed above, 50 mm petri dishes and a millipore filter system are required.

96-Hour Experiments

Decant most of the water from a stock *Ctenodrilus* jar. Pour the remaining water into one or more petri dishes. Examine under a dissecting microscope. Because *Ctenodrilus* is dark purple use a white background to facilitate recognition of the various stages. Use only those specimens that are short (Figure 17) and are not undergoing the early stages of formation of reproductive buds (Figure 19). The early stages of transverse fission are recognized by the formation of the future prostomia. Transfer all non-reproductive specimens with a pasteur pipette into a separate petri dish. Examine all specimens for injuries and again for early signs of reproduction. Discard any specimens not suitable for the toxicity test. Small plastic petri dishes measuring 50 mm in diameter are the most convenient experimental container, although stender dishes have been used with equal success. Add 20 ml of the appropriate concentration of toxicant to the experimental containers. Place five specimens per container, and use ten dishes per concentration to give a total of 50 worms per concentration. Examine daily and record the deaths. Discard all worms at the end of the experiment.

Death in *Ctenodrilus*--

Because of its minute size, death in *Ctenodrilus* is sometimes difficult to ascertain. A healthy *Ctenodrilus* is usually moving; therefore, the condition of these worms is easily distinguished. However, a moribund *Ctenodrilus* usually does not move, and it is necessary to observe such a specimen for a few moments. A dead *Ctenodrilus* usually appears swollen and may be stuck to the bottom of the container. It is possible for *Ctenodrilus* to be completely decomposed within a 96-hour period, so it is necessary to count living specimens. Be sure to check the water-air interface since *Ctenodrilus* has the tendency to crawl to the surface of the water.

Long-term Experiments through Reproduction

Collect the specimens and set up as outlined for the 96-hour toxicity test. Add five pasteur pipette drops of the *Enteromorpha* sp. powder-seawater mixture to each experimental container. Solutions should be renewed every four days, but because of the small size of *Ctenodrilus* the number of specimens within each container should be counted before and after each change. At the end of the experimental period, usually 28 days, count the number of specimens in each container and record. In addition, note the number of specimens undergoing bud formation (Figure 19). This latter observation may be of value in the interpretation of data. Discard all specimens at completion of the experiment.

It is possible to combine the 96-hour and reproductive experiment into one experiment. In this case set up the experiment as described for the 96-hour one. After examination for deaths at 96 hours, add food to each container in the concentration as noted above and continue the test.

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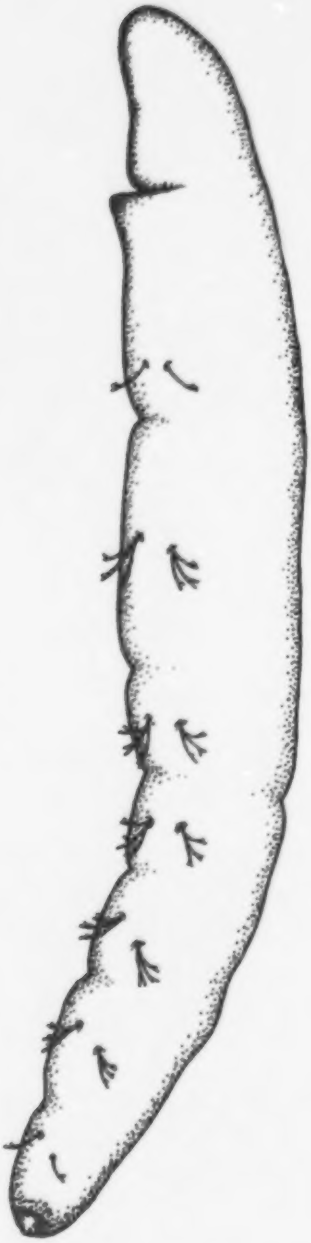

Figure 17. *Ctenodrilus serratus*, dorsal view of entire worm.

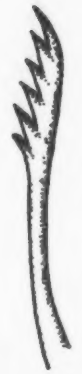

Figure 18. *C. serratus*, simple seta.

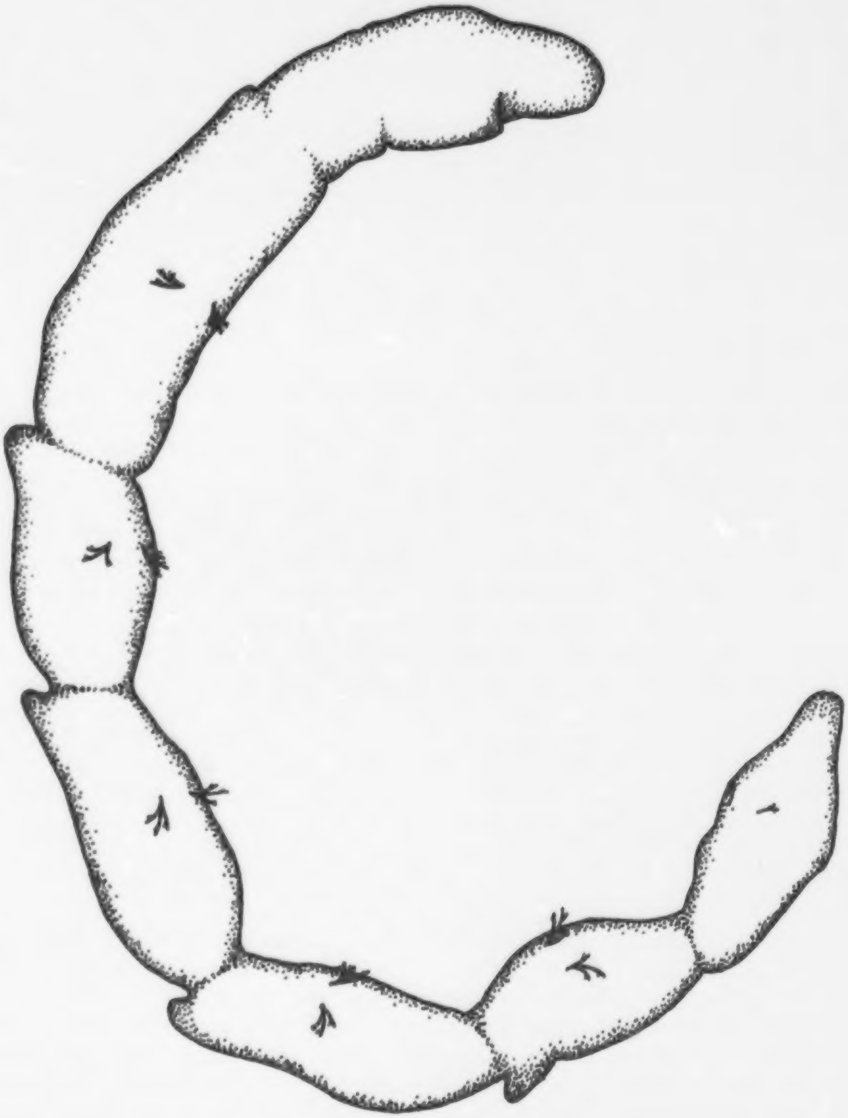

Figure 19. *C. serratus* with five buds forming by transverse fission.

APPENDIX D

CULTURE AND BIOASSAY PROCEDURES FOR POLYCHAETOUS ANNELIDS: *Ophryotrocha diadema* and *Ophryotrocha puerilis*

CULTURE TECHNIQUES

Equipment and Supplies

The equipment and supplies required for a continuous supply of *Ophryotrocha diadema* and *O. puerilis* at the rate of 1,000 specimens each per month are minimal. Approximately one m^2 of shelf space is needed for each species. A constant temperature room is useful for culturing these species since best results have been obtained at 19±1°C. Specimens will reproduce successfully in normal saline water (35 ^{o}/oo). Salinity tolerances have not been investigated. Both species of *Ophryotrocha* are cultured in a static system. Approximately 1,000 liters of seawater are required per month to culture both. A compressed air system is the most convenient method to supply air to each aquarium, although two air pumps could supply sufficient air for these cultures. A dissecting microscope with a good light source is needed to examine the condition of the specimens. Supplies for culturing include 3.78 ℓ (1 gal) jar, pasteur pipettes, petri dishes, 12 cm diameter plastic or glass covers for jars, forceps, plastic tubing, air stones, and a microporous filter (45 μ) system. Food consists of a powder prepared from commercial fish food such as Biorell.

Life History

The genus *Ophryotrocha* is a minute polychaete that is widespread throughout temperate regions of the world. It is difficult to observe in the field because of its size. The most convenient way to collect *Ophryotrocha* is to bring clumps of fouling organisms from boat floats or pilings into the laboratory and place them in a pan of seawater. After a period of time, examine the animals that crawl up along the side of the pan under the microscope, and look for *Ophryotrocha* (Figure 20). Remove with an extra fine brush, and place in a petri dish containing seawater.

Species of the genus *Ophryotrocha* are very difficult to identify and require the help of an expert. Historically, all species of *Ophryotrocha* were referred to as *O. puerilis*, but the experimental studies of several European workers have indicated that several species are involved. This species was originally collected from Los Angeles Harbor. Living specimens were sent to Dr. Bertil Åkesson in Sweden. He identified both *O. labronica* and an unidentified species from this material that he described as *O. diadema* (Figure 20).

Specimens of *O. puerilis* are a subculture of a colony maintained in Sweden by Åkesson. They were brought to Long Beach by a colleague of Åkesson in 1974.

Members of the genus *Ophryotrocha* are either protandric hermaphrodites or dioecious. *Ophryotrocha diadema* belongs to that group of species that are protandric hermaphrodites with a restrictive number of male anterior segments and a restrictive number of female posterior segments. Presumably, self fertilization does not occur. The various stages in the life cycle of *O. diadema* are illustrated in Figures 21-24.

Positive identification of the species of *Ophryotrocha* depends upon a knowledge of the reproductive biology of the species. Species are separated on the basis of being either monecious or dioecious, the number of eggs, the nature of the egg capsule, if present, and the chromosome number.

Techniques of Handling *Ophryotrocha*

The techniques described herein apply equally to both *O. diadema* and *O. puerilis*; however, the life cycle is shorter for *O. diadema*. Any plans for culturing large quantities of these two species for experimentation must take this difference into account. Since these species have such short life histories and since the early stages of development take place within the egg capsule, it is not necessary to use special techniques with the different stages. Stock colonies should be maintained at 19±1°C. Add 2,500 ml of filtered seawater to a 3.78 ℓ jar. Best results are obtained when the filtered seawater is additionally filtered through a microporous filter system. Provide aeration with an air stone connected with plastic tubing to an air supply. Cover the jar with a plastic lid. Place 20-50 specimens per jar and feed with about 0.05 g of powdered Biorell about every 10-14 days. Åkesson uses frozen spinach as a food supply for his cultures of *Ophryotrocha*. The spinach is fragmented in a mixer and then washed several times with seawater. The suspension of spinach in seawater is then fed to the cultures. Since these species reproduce rapidly, the population reaches its peak in about three to five weeks, then declines. Each jar should be reestablished every four to five weeks. The reproductive capacity of *Ophryotrocha* can be slowed by transferring the jars to a colder temperature (either a cold room or a cold bath). It is possible to delay reestablishment of the jars by as much as two to three months if the colony is maintained at 15-16°C.

Problems in Culturing--

Most of the difficulty in culturing *Ophryotrocha* can be attributed to the lack of regular care, especially the neglect of reestablishing new cultures at four to five weeks. Records of the date of establishment of each jar should be kept to minimize this potential problem. A fungal growth appears whenever the colony is overfed. This fungal growth can lead to a poor yield or loss of the colony within the jar. If the fungal growth does appear, the colony should be reestablished. It is especially advantageous to use microporous-filtered seawater in all jars to minimize growth of microorganisms.

Enemies--*Ophryotrocha* are generally parasitized with gregarines (Protozoa:Sporozoa). While these internal parasites are not lethal to the host, they lead eventually to a decrease in vitality. The buildup of the gregarines generally occurs in jars that have been established for five weeks or longer. Regular care will minimize the problem but not eliminate it.

TOXICITY TEST PROCEDURES

Equipment and Supplies

Stender dishes are needed to conduct toxicity tests with *Ophryotrocha*.

96-Hour Experiments

Decant all but 100 ml of seawater from a stock jar. Transfer the remaining water into petri dishes, and place them under the dissecting microscope for examination and selection of test organisms. Remove only the smaller sized specimens and place in a separate petri dish. Place five specimens in a stender dish together with 25 ml of seawater and the toxicant. Use ten dishes per concentration and a minimum of five concentrations plus controls. At the end of the 96-hour test period count the number of living specimens in each container and record. Discard all specimens at the end of the experiment.

Long-Term Experiment

Follow the identical procedures as outlined for the 96-hour bioassay experiment. In fact, the same experiment can serve as both the short-term and long-term experiment. At 96 hours, add to each dish five pasteur pipette drops of a suspension of a 1 percent solution (by weight) of Biorell powder in seawater. Solutions should be renewed every four days, but because of the small size of *Ophryotrocha*, the number of specimens within the dish should be counted before and after each change. After 28 days, count the number of specimens in each jar. A separate count can be made of the number of eggs or larvae within egg capsules. Discard all specimens at the termination of the experiment.

Death in *Ophryotrocha*--

Because of its small size, it is usually difficult or impossible to detect sick or dying specimens. The lack of fecal pellets on the bottom of the dish in long-term experiments is an indication of either death or distressed specimens. Dead *Ophryotrocha* are generally found on the bottom of the dish. If decomposition has occurred, then only the black jaws can be seen.

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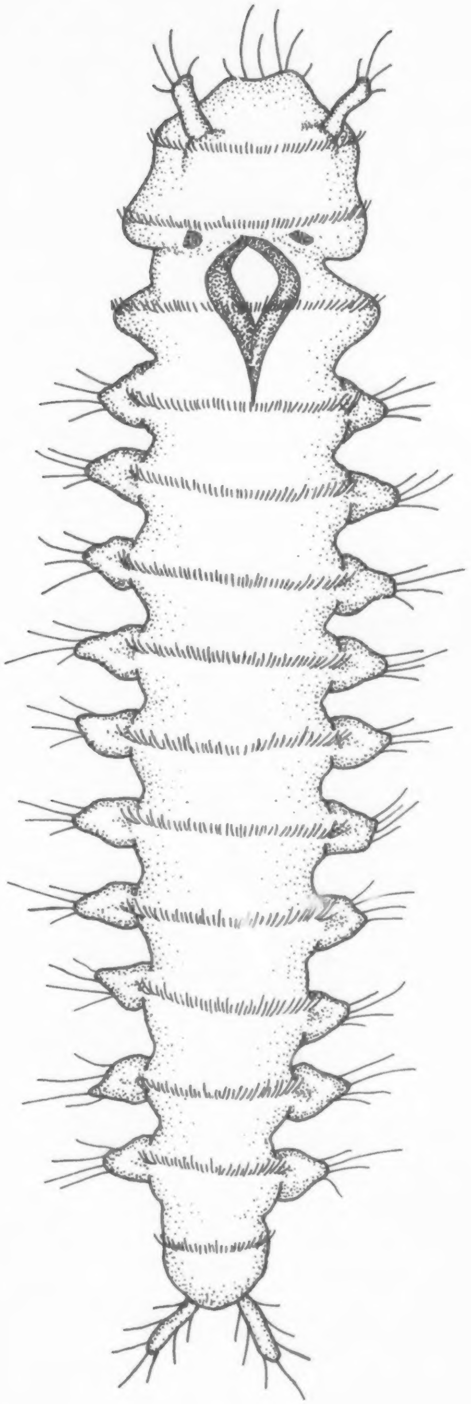

Figure 20. *Ophryotrocha diadema*, dorsal view of entire worm (after Åkesson, 1976).

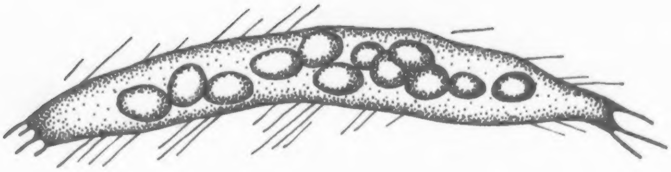

Figure 21. *O. diadema*, egg mass (after Åkesson, 1976).

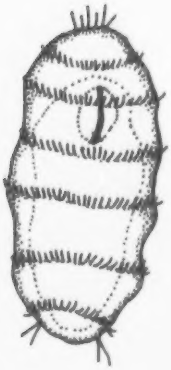

Figure 22. *O. diadema*, larva from egg mass (after Åkesson, 1976).

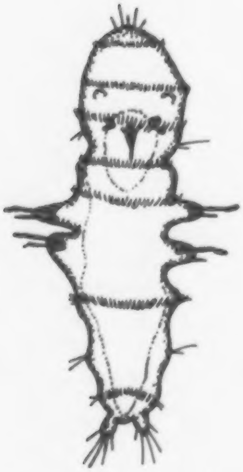

Figure 23. *O. diadema*, larva from egg mass (after Åkesson, 1976).

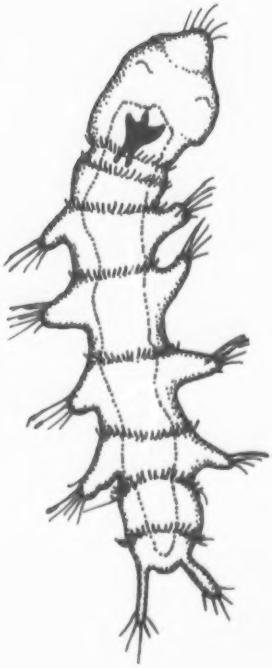

Figure 24. *O. diadema*, released larva (after Åkesson, 1976).

APPENDIX E

CULTURE AND BIOASSAY PROCEDURES FOR POLYCHAETOUS ANNELID: *Janua (Dexiospira) brasiliensis*

CULTURE TECHNIQUES

Equipment and Supplies

The equipment required for a continuous supply of *Dexiospira* consists of a dissecting and compound microscope, a good microscope light, and a blender. Approximately two m^2 of shelf space is required to culture about 1,000 specimens. A constant temperature laboratory maintained at 19±1°C will insure maximum results since this species is especially sensitive to temperatures above 21° or 22°C.

Natural, filtered seawater at a salinity of 35.5 $^o/oo$ will yield the maximum number of offspring since this species is sensitive to salinities below about 32 $^o/oo$. Approximately 100 liters of seawater are required per month to culture a population of 1,000 to 2,000 specimens. A central compressed air system is the most convenient source of air for the culture. It is advantageous to filter all seawater to be used in culturing *Dexiospira* through a microporous filter (45 μ) to minimize the growth of microorganisms. Additional supplies required for culturing *Dexiospira* include 3.78 ℓ (1 gal) glass jars, petri dishes, pasteur pipettes, beakers, forceps, microscope slides, plastic string, air stones, plastic tubing, and scalpel. Since *Dexiospira* requires living food (*Dunaliella tertiolecta*), the techniques, equipment, and supplies required to culture this phytoplanktonic organism are given as a separate section within Appendix E.

Collecting Techniques and Life History

Dexiospira occurs in abundance on floating boat docks in southern California. This species grows on the surface of docks as well as fouling organisms; it is easily collected from the surface of mussels (*Mytilus edulis*) or sea lettuce (*Ulva lactuca*). The calcareous tube of this species is white, coiled dextrally (counter-clockwise), and measures one to two mm in diameter (Figure 25). Sinistral tubes of spirorbids and other dextrally coiled species may be found in association with *Dexiospira* so careful identification is required.

While *Dexiospira* is widespread only in the warmer waters of the world, the techniques described herein could apply to other spirorbid polychaetes. Identification of the Spirorbinae is extremely difficult. The key characteristics employed in identification are as follows: orientation and morphology of the tube, method of brood protection, number of thoracic segments, and types of setae and uncini. As the setation is very similar in many species, it may be necessary to send specimens to an authority for species confirmation. Consult the papers by Knight-Jones and others (1959, 1975, 1977) for keys to the species of spirorbids.

The tubes of *Dexiospira* are easily removed from the fronds of *Ulva* with a scalpel. If they are found on mussel valves, it is better to leave them on, since many of the tubes would be crushed on removal. It is best to open the mussel and remove all of the soft tissue. This will prevent possible future fouling of the water by the mussel. The suspension of glass jars or wooden blocks from a boat dock for about a month is a convenient method of collection.

Animals removed from *Ulva* should be placed in glass petri dishes using a pasteur pipette with an adequate opening. The cleaned mussel valves should be placed in 3.78 ℓ (1 gal) jars. If the wooden blocks are small enough, these also may be placed in small aquaria.

Dexiospira is hermaphroditic. Although these animals are capable of self-fertilization, the viability of the embryos seems to decrease with repeated self-fertilization. Therefore, it is advantageous to culture them in groups rather than separately. After fertilization, the embryos are transferred (method unknown) to the operculum, which is modified as a brood chamber (Figure 26). The brooding period lasts from six to eight days and is followed by the discharge of the pelagic larvae. The larvae settle and begin tube formation within one hour following their release. Sexual maturity is attained in 30 days.

Techniques of Handling *Dexiospira*

Place a petri dish containing 50 to 100 tubes of *Dexiospira* on the bottom of a 3.78 ℓ jar provided with aeration and containing 2,500 ml of millipore-filtered seawater. Suspend microscope slides (2.54 x 7.62 cm) in the water in order to provide a surface for larval settlement. In order to facilitate larval settlement prepare the slides as follows: place fresh *Ulva* in a blender and grind the alga into small pieces. Pour the material into a beaker, and allow the larger pieces to settle. Dip a clean microscope slide into the supernatant fluid for a moment and remove. It is not necessary to dry the slide; it can now be suspended in the jar with a plastic string. Since field specimens are reproductively active year round, only two to three days are required for adequate larval settlement on the glass slides. Transfer the slides to a clean aquarium provided with aerated seawater and *Dunaliella* as food. A new set of slides can be placed in the original aquarium for additional settlement. This procedure can continue until the field collected specimens are no longer in the reproductive state.

The green phytomastigophoran *Dunaliella* has been found to be the best food source. Approximately 30 to 40 ml of *Dunaliella* are added to each aquarium twice a week to maintain the culture. The seawater should be changed every two to three weeks.

If the mussel valves with tubes are not properly washed of extraneous material, the culture will be infested with a myriad of unnecessary organisms. These are not necessarily harmful to *Dexiospira*, but they will cause an increase in the amount of *Dunaliella* required to maintain the culture. This contamination can be reduced by changing the water every two to three weeks and by using microporous-filtered seawater. Larvae of *Dexiospira* will often settle and metamorphose on the surface tension when cultured in petri dishes. They can be dropped to the bottom by touching them with forceps. Larvae will settle on the inside of the aquarium as well as on the glass slides. Allow these specimens to mature, and then they can be removed and utilized in establishing additional colonies. There are no known enemies of *Dexiospira*.

TOXICITY TEST PROCEDURES

Only small plastic petri dishes (15x60 mm) are required in addition to the equipment and supplies specified above.

96-Hour Experiments

Remove specimens from either the sides of the stock aquaria or from the suspended glass slides. The specimen can be removed with the flanged tipped pipette by scraping the sides with the tip while producing suction with the rubber bulb. There is only a minimal amount of damage to the animal when this technique is employed. Examine each specimen under the dissecting microscope, and discard any injured specimens. Place five specimens in each small petri dish containing 15 ml of seawater and toxicant. Use ten dishes per concentration with a minimum of five concentrations plus control. These worms will not require food during the 96-hour experimental period. Count the number of living worms at 96 hours, and discard all specimens.

Long-Term Experiment Involving Reproduction

Establish the toxicity test as outlined under the 96-hour experiment. Add 1.0 ml of *Dunaliella* to each container every other day, and change the medium in the petri dish once a week during the experimental period. If this procedure is not followed, then a population of microorganisms, in addition to *Dunaliella*, will build up rapidly. Allow the experiment to run 28 to 35 days to allow sufficient time for reproduction to occur. Count the number of living specimens in each container. Mount each specimen on a slide, examine under a compound microscope, and count the number of embryos within the operculum. Discard all specimens at the completion of the experiment. It is possible to combine both the 96-hour LC_{50} experiment and the reproductive experiment. Examine for deaths at 96-hours, record, feed with *Dunaliella*, and allow the experiment to continue for the 28-35 days.

Death in *Dexiospira*--

An animal may be considered dead when there is no sign of movement or when the body is decomposed. It may be necessary to open the tube to determine the animal's condition. The tube can be opened by tapping the tube with a pair of fine forceps.

LABORATORY CULTURE OF *Dunaliella tertiolecta*

Medium

Seawater: Use about 30-32 $^{o}/oo$ salinity seawater (90% seawater and 10% double distilled water). Filter through a microporous-filter system (45 µ). For 50 ml quantities, mix complete medium (below) and autoclave at 15 psi for 15 minutes. For larger quantities, autoclave phosphate separately, and then add to the medium. If a serious precipitation occurs, it may be necessary to filter sterilized medium prior to use.

Major nutrients: Nitrate-- 5 g KNO_3 made to 1,000 ml with H_2O. This stock solution may be stored in a refrigerator for up to four months. Add 1.0 ml stock solution to one liter distilled water to make a final solution; use ten ml of this solution per liter of culture medium. Do not store final solution.

Phosphate--0.68 g KH_2PO_4 made to 1,000 ml with H_2O. This solution may be stored in a refrigerator for up to four months. Add 1.0 ml stock solution to one liter distilled water to make a final solution; use ten ml of this solution per liter of culture medium. Do not store final solution.

Minor trace metals--Stock metals--30 mg $ZnSO_4 \cdot H_2O$, plus 25 mg $CuSO_4 \cdot 5H_2O$, plus 20 mg $CuSO_4 \cdot 7H_2O$ dissolve 5.0 g $FeCl_3 \cdot 6H_2O$ and 2.0 g $MnSO_4 \cdot H_2O$ in 1,000 ml distilled water (ignore slight precipitate).

Sodium Molybdate--25 mg $Na_2MoO_4 \cdot H_2O$ in 1,000 ml of distilled water.

Sodium Ethylenediaminetetraacetate--Dissolve 50 g of Na_2E.D.T.A.$\cdot 2H_2O$ in 1,000 ml of distilled water.

Metal Mixture--Add 100 ml of the Na_2E.D.T.A.$\cdot 2H_2O$ solution and ten ml each to the three minor trace metal solutions listed above to about 800 ml of distilled water. Adjust the pH to 7.5 with dilute NaOH solution and make up to 1,000 ml with distilled water. Add 1.0 ml to each liter of culture medium. This solution may be stored for up to four months in a refrigerator.

Vitamins: Vitamin B_{12}--Dissolve 10.0 mg crystalline vitamin B_{12} in 100 ml of distilled water. Store in deep freeze until use. Immediately prior to use, thaw solution, remove 1.0 ml and refreeze stock solution. Dilute the one ml of vitamin B_{12} solution with 99 ml of distilled water. Add 1.0 ml of diluted solution to each liter of seawater and throw away the remainder.

Biotin--Dissolve 10.0 mg biotin in 100 ml of distilled water and freeze. Immediately prior to use, thaw the solution, remove 1.0 ml and refreeze the stock solution. Dilute the 1.0 ml with 99 ml distilled water. Add 1.0 ml of diluted solution to each liter of seawater and throw away the remainder.
Thiamine hydrochloride--Dissolve 100 mg of thiamine hydrochloride in 100 ml of distilled water and freeze. Immediately prior to use thaw, remove 1.0 ml, and refreeze the stock solution. Dilute the 1.0 ml with 99 ml of distilled water. Add 1.0 ml of diluted solution to each liter of seawater and throw away the remainder.

Culture

Place 5 to 15 ml of *Dunaliella* from a previous culture in a 500 ml Erlenmeyer flask containing 500 ml of autoclaved medium. The amount of *Dunaliella* depends upon its concentration in the previous culture. Place stoppered Erlenmeyer flasks under grow-lux fluorescent lights. Depending upon the amount of *Dunaliella* required, the alga can be exposed to continuous light or to a light-dark cycle, which is maintained with an electrical timer. The growth rate can be slowed, if desired, by placing the cultures in a cold bath (17°C) with the lights placed overhead.

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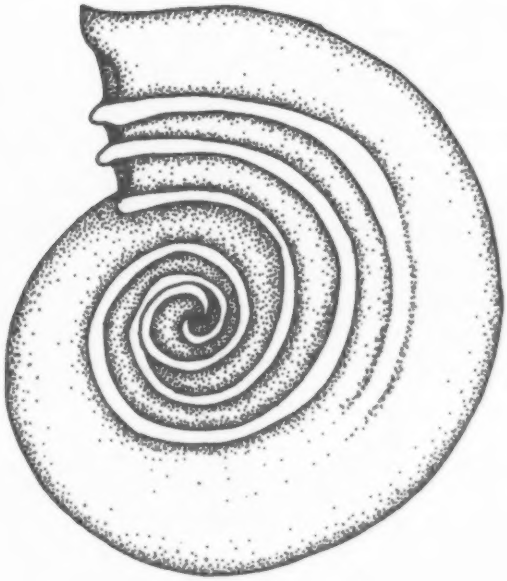

Figure 25. *Janua (Dexiospira) brasiliensis*, tube.

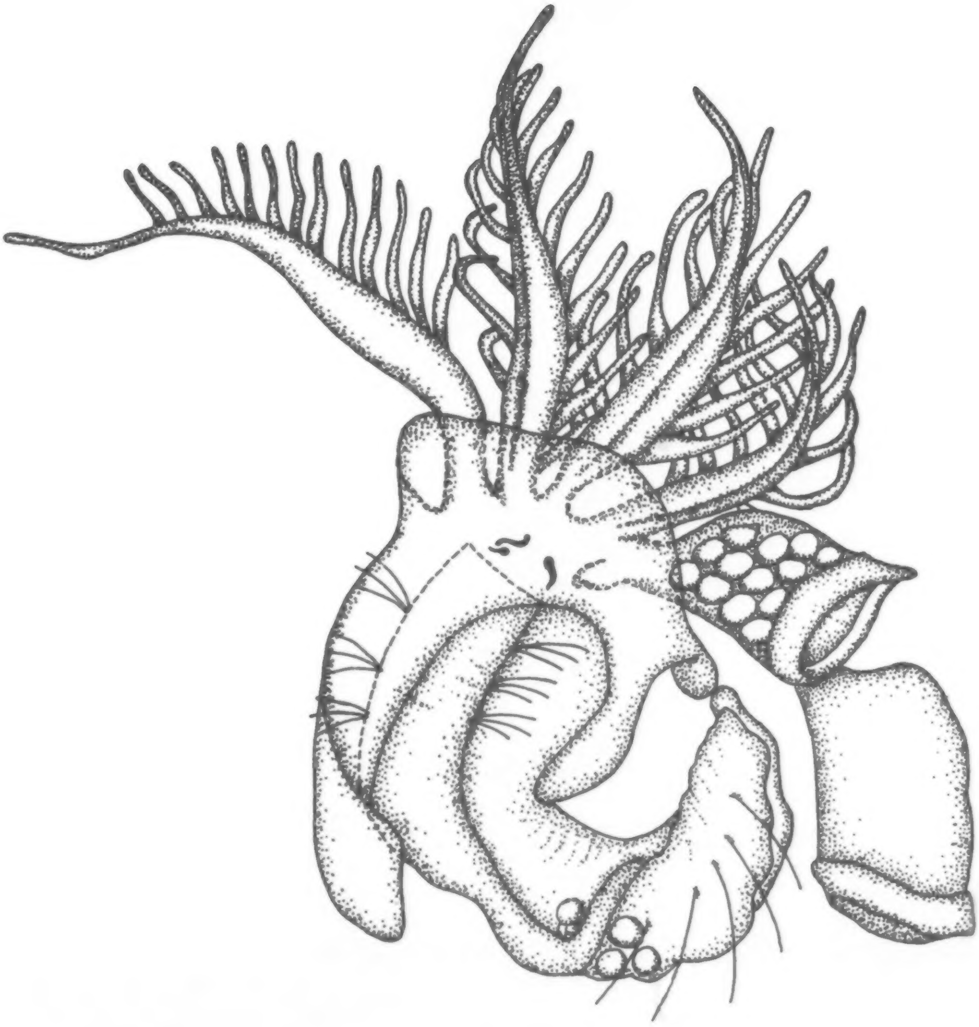

Figure 26. *D. brasiliensis*, dorsal view of entire worm removed from tube.

APPENDIX F

CULTURE AND BIOASSAY PROCEDURES FOR POLYCHAETOUS ANNELIDS: *Polydora ligni* and *Boccardia proboscidea*

CULTURE TECHNIQUES

Equipment and Supplies

The only equipment required to culture these two spionid polychaetes is a dissecting and compound microscope. A blender is convenient to mix food. About four m^2 of shelf space is required to culture a large number of both these species. A constant temperature laboratory set at 19±1°C provides the ideal environment. For best results, filter all seawater through a microporous filter (45 μ) to remove microorganisms; however, stock colonies do not necessarily require it. Approximately 150-300 liters of seawater are required per month to culture these two species. A central compressed air system is the most satisfactory method of supplying air to the aquaria. A magnetic stirring device (Hinegardner, 1969) is convenient for culturing the planktonic stages. Consumable supplies include 3.78 ℓ (1 gal) jars, petri dishes (20 x 100 mm), pasteur pipettes, 400 ml plastic tri-pour beakers with lids, air stones, plastic tubing, and glass tubing.

Collecting Procedures and Life History

Polydora ligni--

This species of polychaete is found throughout the temperate regions of the world and may inhabit substrata ranging from silty mud to sand. The worm is generally found inhabiting tubes made of mud or sand lying within the upper few centimeters of sediment. Since this species has a rather wide salinity tolerance, it may be found anywhere from the open coast to the estuarine environment.

Adult *Polydora* can be collected by use of a 0.5 mm screening box. In the collection area, several scoops of the top two to five cm of the substratum are placed in the screening box and are gently shaken through the screen. The adult worms and their tubes will remain on the screen and can be washed into a container of seawater. Since many of the adults will remain inside their tubes during the sifting process, all tubes left on the screen should be saved and inspected for worms in the laboratory under the dissecting microscope. Adults measure from 3 to 25 mm in length.

If no suitable collection area can be found for the adult worms, a population can be started from the planktonic larvae. *Polydora* reproduces seasonally in some locations and almost continuously in other areas (Rice, 1975). Generally, the planktonic larvae can be obtained by use of a shallow (as near the surface as possible) plankton tow using a fine mesh plankton net. Larvae obtained in this manner will settle out and metamorphose into adults within one to two weeks under laboratory conditions. Place the larvae in a 3.78 ℓ jar provided with 2,500 ml microporous-filtered seawater, some fine sand, and an air supply.

Polydora (Figures 27 and 28) belongs to a subgroup of the Family Spionidae known as the polydorid complex that contains the genera *Polydora*, *Boccardia*, and *Pseudopolydora*. These worms share the common feature in which the setae of their fifth setiger are modified into thick heavy spines of various shapes (Figure 29). This feature is useful in identification of species. *Polydora* is distinguished from the other members of the genus by the presence of the following three structures: 1) a subdistal tooth near the tip of the heavy spines of the fifth setiger (Figure 29), 2) small brush-like companion setae lying directly on top of the heavy spines of the fifth setiger (these can only be seen under a compound microscope) (Figure 29), and 3) a small nuchal antenna on the caruncle just between the palps (Figure 27). For further data and keys to the species of *Polydora* see Blake (1971) and Hartman (1969).

The planktonic larvae of *Polydora* are very difficult to distinguish from the other members of the genus. Once the larvae have settled and metamorphosed into the adult form, the above characteristics may be used for identification. For data on larval morphology and occurrence see Hannerz (1956) and Blake (1969).

Spawning occurs throughout the year under laboratory conditions. The male releases spermatophores out the anterior opening of his tube. The spermatophores are picked up by the palps of the female and transported into her tube where the spermatophore is broken and the sperm released. Fertilization has not been observed, but apparently sperm are stored in the seminal receptacle with fertilization occurring during formation of the egg capsule. Early development, through the three-segmented larval stage, takes place in these egg capsules (Figure 30). Development from the 3 to the 14-segmented stages takes place in the water mass, then the larvae metamorphose and settle (Figures 31 and 32). Sexual maturity is reached in 50 to 60 days.

Boccardia proboscidea--

This species has been found from the intertidal and shallow subtidal waters of British Columbia south to southern California. It constructs sand tubes in the sediments of bays and estuaries, among clumps and holdfasts of rocky intertidal algae, within shells of mollusks, and within soft sandstone rocks. Adult *Boccardia* are collected conveniently by either screening intertidal sediments through a 0.5 mm screening box or finding populations within sandstone rocks. *Boccardia* is recognized by the snout-like prostomium and by the presence of sooty pigmentation along either side of the prostomium (Figure 33). Confirmation of this species depends upon the microscopic examination of the setae of the fifth setigerous segment (Figure 34). Adults measure up to 5 mm in length.

Boccardia produces two different types of eggs: one measures 100 μ in diameter and develops into planktotrophic larvae, and the other egg type measures 150 μ in diameter and develops into lecithotrophic larvae. Additional differences that result from these two egg types are noted below.

In the population of *Boccardia* that lays the smaller egg, the eggs are presumably fertilized with sperm stored in the seminal receptacle, similar to the *Polydora*. The zygotes are deposited in egg capsules (Figure 35), which number one per segment, and may total up to 75 per specimen. The number of zygotes per capsule varies from 4 to 250 with an average of 150. Each zygote produces a larvae that hatches from the capsule. After five days, setae appear on three segments (Figure 36). The setae elongate and the larvae emerge from the capsule after eight days. New capsules are formed as soon as the larvae from the first laying are released. Larvae remain planktonic from day 8 to 29 at which time they possess about 12 to 14 segments. Metamorphosis to the benthic stage was first noted after 29 days with larvae possessing 15 segments (Figure 37). Sexual maturity is reached as early as 110 days.

Development in the population of *Boccardia* that produces the larger egg proceeds somewhat differently with regard to timing of the various stages and the number of young produced. Three setigerous larvae are noted at 96 hours with additional segments appearing more or less daily; a 13-segmented larvae is ten days old. After 17 days, 13 to 15 segmented larvae hatch from the capsule. Only one or a few larvae emerge from a single capsule; the remaining zygotes served as nurse cells during development of these few larvae. This form has not yet been reared to sexual maturity in the laboratory.

Techniques of Handling *Polydora* and *Boccardia*

Polydora ligni

Since the life cycle of this polychaete involves both a benthic and planktonic stage, it is necessary to employ special techniques in handling the adults and larvae. Some of the techniques for handling both stages have been described under collecting techniques above. About 50 to 100 adults can be maintained in 3.78 ℓ (1 gal) jars filled with 2,500 ml of filtered seawater. Aeration is provided with an air stone attached via plastic tubing to a compressed air system. Adults are fed an *Enteromorpha* sp. and Biorell (or Tetramin) food mixture (4:1 ratio). *Enteromorpha* sp. is collected from estuarine areas and washed repeatedly to remove sediment and debris. The *Enteromorpha* sp. is air-dried and stored in plastic bags. The food mixture for *Polydora* is prepared by drying the alga in an oven at 35°C for 15 minutes. Then the crisp *Enteromorpha* sp. is placed in a blender and chopped into a fine powder. This powder is shaken through a screen with a 0.05 mm mesh. The *Enteromorpha* sp. powder is collected on a pan underneath the screen. This food can be stored for an indefinite length of time.

Biorell or any commercially available fish food can be used as a high protein supplement along with *Enteromorpha* sp. The Biorell is prepared by chopping the food in a blender and then shaking it through a 0.06 mm screen. The fine powder is collected from the pan below the finest screen. The food can be stored indefinitely in this form in a moisture proof container.

For best results, 0.25 g of food mixture should be added to each jar twice a week, and the water should be changed once a month. Larger numbers of individuals can be maintained in 18.9 ℓ aquaria using similar techniques. A good indication of a healthy culture is the presence of the worm's palps extending out of the end of its tube in search of food. Also, it is often possible to directly observe the worms in their tubes by use of a hand lens when the tubes are constructed on the side of the jar.

Planktonic larvae may periodically appear, usually in large numbers in the adult culture aquaria. The larvae are small and hard to see, but by turning off the air supply and directing a strong beam of light through the container, the swimming movements of the larvae can be observed. The larvae are strongly attracted to light in their early stages, and they can be concentrated and removed from the adult colony by directing a light through the aquarium near the surface of the water. Within 10 minutes, nearly all of the larvae will have accumulated at the point nearest the light source and they can be removed with a pipette. Most field-collected or laboratory-cultured larvae will not survive to maturity. Generally, less than 10 percent will reach adulthood. For maximum survival, the larvae should be placed in about 300 ml of microporous-filtered seawater in an appropriate container, such as a plastic Tri-pour beaker with a perforated cardboard cover, and provided with about 20 ml of *Dunaliella* as a food source twice a week. Put about 300 larvae in each container. Then place the containers onto a magnetic stirring device (Hinegardner, 1969). Within two or three weeks the larvae will settle on the bottom and metamorphose into the adult form. The metamorphosed adults can be kept for two or three months in the same beakers. When it is observed that most of the larvae have metamorphosed into adults, their diet should be changed to the *Enteromorpha*-biorell mixture and the water in each container changed every two weeks.

Boccardia proboscidea--

The same techniques used in culturing the adults and larvae of *Polydora ligni* are used in culturing *Boccardia*.

Problems in Culturing *Polydora* and *Boccardia*--

The primary problem in culturing these adult spionids is overcrowding and overfeeding. Since these species may be present in large numbers in the field (10,000 to 50,000 per m^2 or more), the tendency is to attempt to maintain all collected specimens. This results in overcrowding that in turn will result in death of some specimens. These deaths will foul the water and lead to additional deaths. If too much food is added to the culture, the worms will begin to show stress as a result of lowered dissolved oxygen and leave their tubes. When this is observed, the water in the container should be changed immediately and the worms allowed to recover for one to two days before feeding again. Another common problem occurs as a result of too much Biorell in the food mixture. Large amounts of Biorell, if left uneaten in the culture, will develop a white mold after a few days and may cause the worms to leave their tubes. Again, this problem can be alleviated by changing the water and reducing the amount of Biorell in the food.

Maximum survival of larvae depends largely upon keeping the cultures as free from contamination as possible. The most common source of contamination is the presence of an adult culture of protozoans and bacteria. These organisms do not harm the adults but may be deleterious to the larvae. It is often helpful to put the larvae through several changes of microporous-filtered seawater in small petri dishes (use the light attraction technique described above to concentrate the larvae) before adding the *Dunaliella*.

TOXICITY TEST PROCEDURES

Equipment and Supplies

No additional equipment or supplies are required to conduct toxicity tests with *Polydora ligni* and *Boccardia proboscidea*; however, most of the studies have been conducted with *Polydora*.

Remove the required number of individuals from their tubes in the stock culture and transfer with a pipette to a glass petri dish. Examine all specimens under the dissecting microscope for injured or reproductive individuals. Discard all injured worms, and return reproductive specimens to the stock colony. Maturing males usually appear milky white, posterior to setiger 16, and females appear grey to pink when eggs are present. Place 15 to 20 ml of the desired toxicant solution in each 60 x 15 mm petri dish. One or two worms can be kept in each dish. Use a minimum of 20 worms per concentration. Use a total of five concentrations plus control. Add a small amount (0.14) of fine sand (diameter 0.125-0.25 mm) to each dish as material for tube construction. Examine for deaths daily (see below) and discard all worms at completion of the experiment.

Long-Term Experiments (28 days)

Follow the same procedures as outlined for the 96-hour bioassay. Since both species begin to show stress in about seven days if they have not been fed or do not have materials for tube construction, it will be necessary to feed the worms weekly. Provide each dish with approximately 0.1 g of fine sand or silt for the tube construction material. Feed five pasteur pipette drops of the *Enteromorpha*-Biorell mixture (0.05 g) suspended in microporous-filtered seawater per dish. The water should be renewed in each dish prior to the weekly feeding. The condition of each worm should be determined prior to feeding each week. If no movement can be seen (usually the palps move about and can be easily seen with the unaided eye after some experience), decant the fluid, invert the dish, examine the tube under the dissecting microscope, and note the health of the worm within its tube.

Death in Adult *Polydora* and *Boccardia*--

Stressed spionids generally abandon their tubes, which will ultimately lead to their death. While normal healthy spionids can construct a new tube, a stressed specimen cannot. Once the specimen abandons its tube, it may fragment, lose its palps, and be attacked by microorganisms. It is important in both the 96-hour and 28-day bioassay experiment to note whether or not the specimen is within its tube since this can be an indication of sublethal effects.

96-Hour Toxicity Tests with Larvae

Only limited success has been obtained thus far in conducting bioassays with *Polydora* larvae; no tests have been carried out with *Boccardia*. Because of the large number of larvae produced and because of the ease in concentrating and collecting them with a light, the use of the larvae of these species have a potential for being a useful larval bioassay organism if techniques can be developed to greatly increase the survival rate.

Concentrate the larvae with a light, and pipette them into a petri dish. Place 10 larvae in a petri dish containing toxicant. Use 10 dishes per concentration and a minimum of five concentrations plus control. Examine for living larvae at 96 hours; dead ones may have decomposed by 96 hours. Since many specimens may die during the experimental period, at least 85 percent of the larvae should be living within the control to be considered a valid experiment. Discard all specimens at the termination of the experiment.

Long-Term Experiments through Reproductive Period

No toxicity tests through a reproductive period have been conducted thus far. The procedures outlined herein are intended to serve as a guide to conduct such an experiment. Place ten worms in a 100 x 20 mm petri dish together with microporous-filtered seawater and 0.1 g of powdered sand or clay. Allow 24 hours for the specimens to construct a tube. Remove all worms that failed to construct a tube. Decant off all seawater, and fill with the desired toxicant solution. Since it is quite possible that a different number of specimens will be present in each petri dish, a count of specimens should be made so as to insure the same total number of specimens in each concentration. There should be a minimum of five specimens per dish to insure fertilization of eggs. The water should be changed and the worms fed at weekly intervals as described for long-term adult bioassay experiments above. Each dish should be checked every four days for the presence of egg capsules (Figures 20 and 25) inside the tubes of the female worms. This can be done by decanting the water and inverting the bottom of the petri dish on the stage of the dissecting microscope and examining each tube for egg capsules. The worms can be exposed to air for up to five minutes without damage. The number of capsules is noted and the average number of eggs per capsule estimated. After a period of several weeks, the reproductive activity for each concentration is expressed as total number of capsules and/or eggs produced per female per concentration.

LABORATORY CULTURE OF *Dunaliella tertiolecta*

Medium

Seawater: Use about 30-32 $^{o}/oo$ salinity seawater (90 percent seawater and 10 percent double distilled water). Filter through a microporous filter system (0.45 μ). For 50 ml quantities, mix complete medium (below) and autoclave phosphate separately and then add to the medium. If a serious precipitation occurs, it may be necessary to filter sterilized medium prior to use.

Major nutrients: Nitrate-- 5 g KNO_3 made to 1,000 ml with H_2O. This stock solution may be stored in a refrigerator for up to four months. Add 1.0 ml stock solution to one liter of distilled water to make a final solution; use ten ml of this solution per liter of culture medium. Do not store final solution.

Phosphate-- 0.68 g KH_2PO_4 made to 1,000 ml with H_2O. This solution may be stored in a refrigerator for up to four months. Add 1.0 ml stock stolution to one liter distilled water to make a final solution; use ten ml of this solution per liter of culture medium. Do not store final solution.

Minor trace metals-- Stock metals--30 mg $ZnSO_4 \cdot 7H_2O$, plus 25 mg $CuSO_4 \cdot 5H_2O$, plus 20 mg $CuSO_4 \cdot 7H_2O$ dissolved in 1,000 ml of distilled water.

Iron + Manganese-- Dissolve 5.0 g $FeCl_3 \cdot 6H_2O$ and 2.0 g $MnSO_4 \cdot H_2O$ in 1,000 ml of distilled water (ignore slight precipitate).

Sodium Molybdate-- 25 mg $Na_2MoO_4 \cdot 2H_2O$ in 1,000 ml of distilled water.

Sodium Ethylenediaminetetraacetate-- Dissolve 50 g of Na_2E.D.T.A.$\cdot 2H_2O$ in 1,000 ml of distilled water.

Metal Mixture-- Add 100 ml of the Na_2E.D.T.A.$\cdot 2H_2O$ solution and ten ml each to the three minor trace metal solutions listed above to about 800 ml of distilled water. Adjust the pH to 7.5 with dilute NaOH solution and make up to 1,000 ml with distilled water. Add 1.0 ml to each liter of culture medium. This solution may be stored for up to four months in a refrigerator.

Vitamins: Vitamin B_{12}--Dissolve 10.0 mg crystalline vitamin B_{12} in 100 ml of distilled water. Store in deep freeze until use. Immediately prior to use, thaw the solution, remove 1.0 ml, and refreeze the stock solution. Dilute the one ml of vitamin B_{12} solution with 99 ml of distilled water. Add 1.0 ml of diluted solution to each liter of seawater and then throw away remainder.

Biotin-- Dissolve 10.0 mg biotin in 100 ml of distilled water and freeze. Immediately prior to use, thaw the solution, remove 1.0 ml, and refreeze the stock solution. Dilute the 1.0 ml with 99 ml distilled water. Add 1.0 ml of diluted solution to each liter of seawater and throw away the remainder.

Thiamine hydrochloride-- Dissolve 100 mg of thiamine hydrochloride in 100 ml of distilled water and freeze. Immediately prior to use thaw, remove 1.0 ml. and refreeze the stock solution. Dilute the 1.0 ml with 99 ml of distilled water. Add 1.0 ml of diluted solution to each liter of seawater and throw away the remainder.

Culture

Place 5-15 ml of *Dunaliella* from a previous culture in a 500 ml Erlenmeyer flask containing 500 ml of autoclaved medium. The amount of *Dunaliella* depends upon its concentration in the previous culture. Place stoppered Erlenmeyer flasks under grow-lux fluorescent lights. Depending upon the amount of *Dunaliella* required, the alga can be exposed to continuous light or to a light-dark cycle, which is maintained with an electrical timer. The growth rate can be slowed, if desired, by placing the cultures in a cold bath (17°C) with the lights placed overhead.

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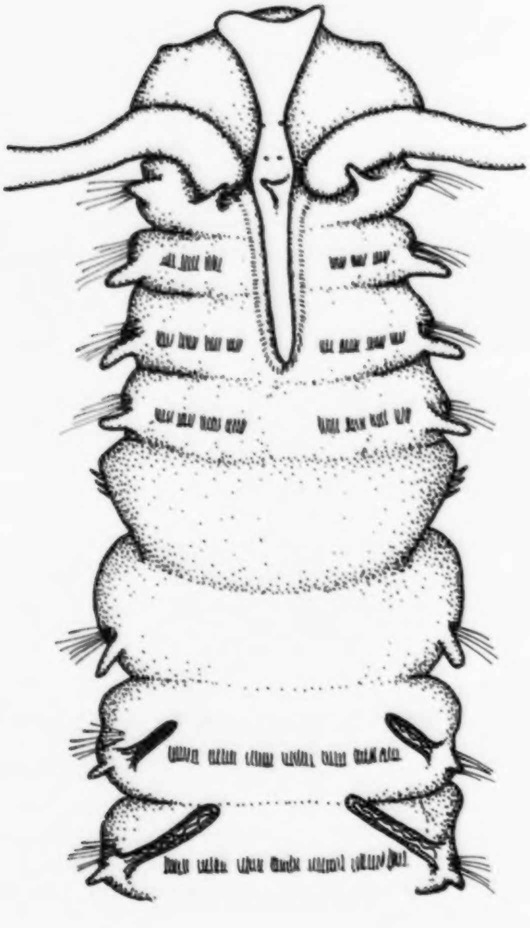

Figure 27. *Polydora ligni*, anterior end, dorsal view showing location of palps, nuchal antenna, and fifth setiger.

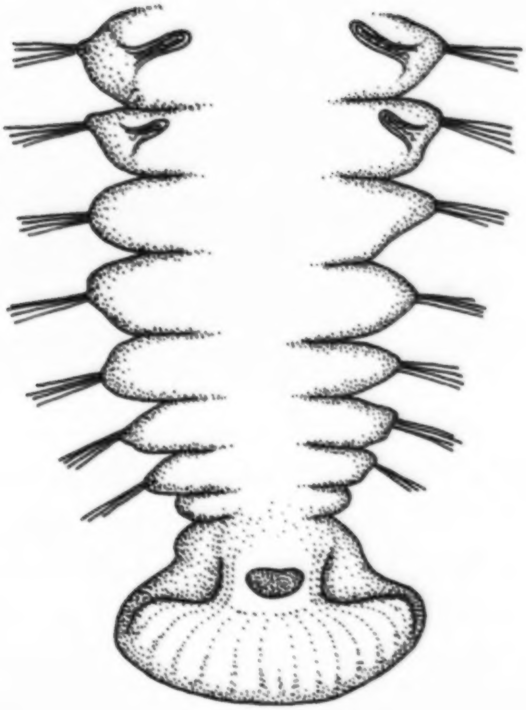

Figure 28. *P. ligni*, posterior end, dorsal view showing disc-like pygidium.

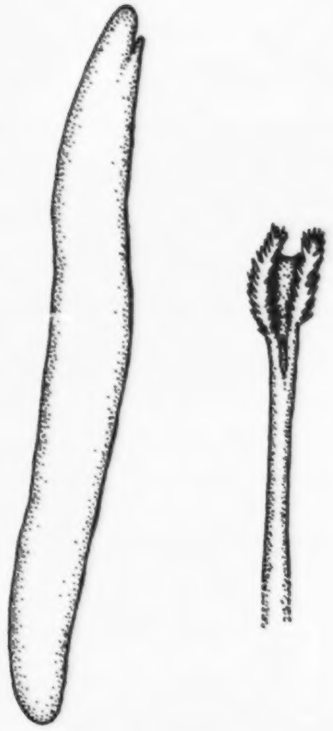

Figure 29. *P. ligni*, setae of modified fifth setiger showing one spine with a subapical tooth and companion seta with forked tip.

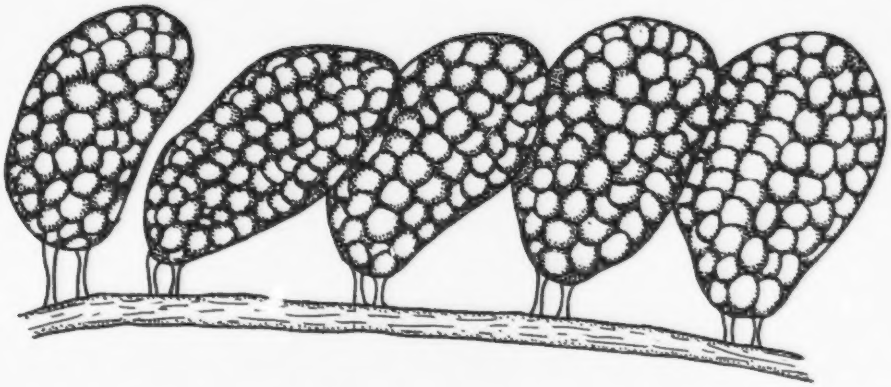

Figure 30. *P. ligni*, portion of a string of egg capsules as they appear inside the tube of the female.

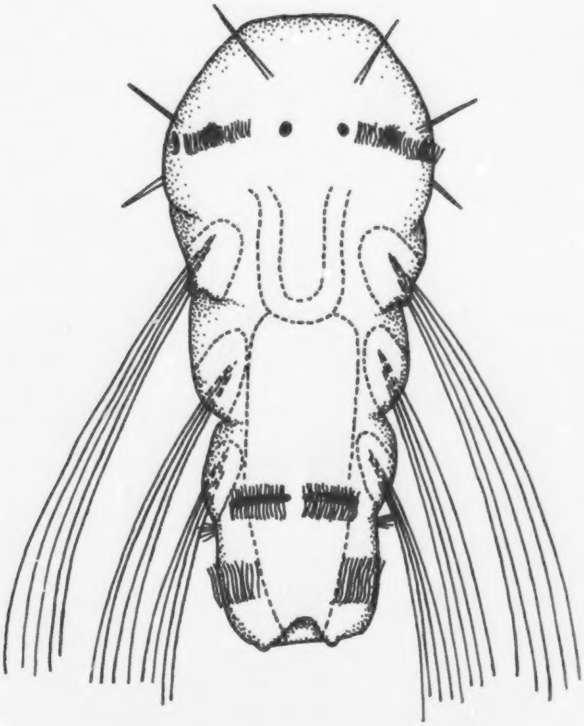

Figure 31. *P. ligni*, three setiger larva just after release from the female's tube.

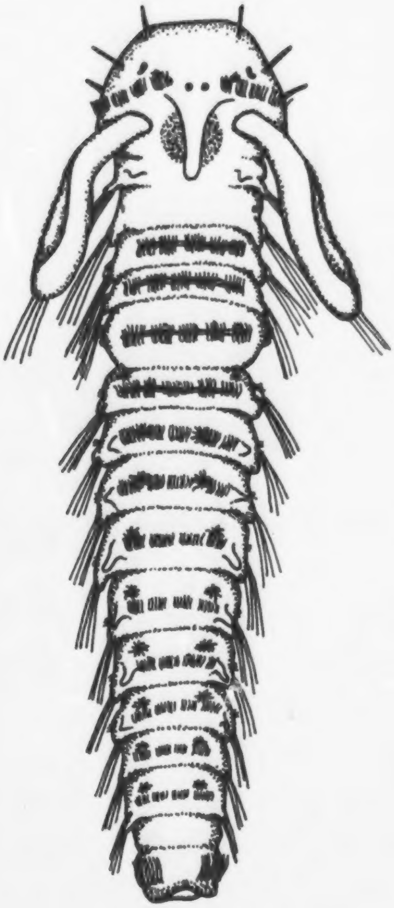

Figure 32. *P. ligni*, 14 setiger larva ready to metamorphose into the adult form.

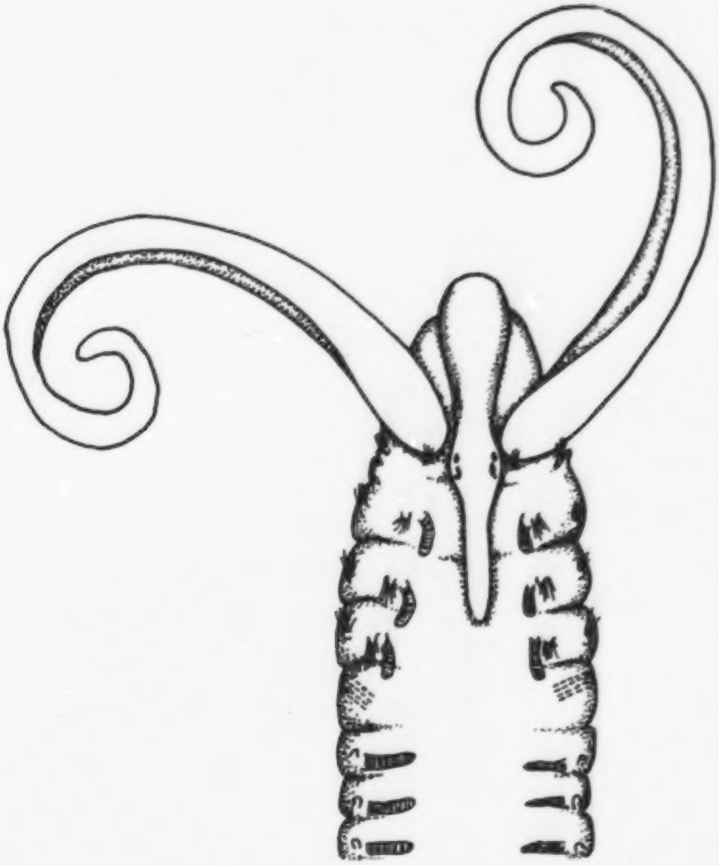

Figure 33. *Boccardia proboscidea*, anterior end, dorsal view.

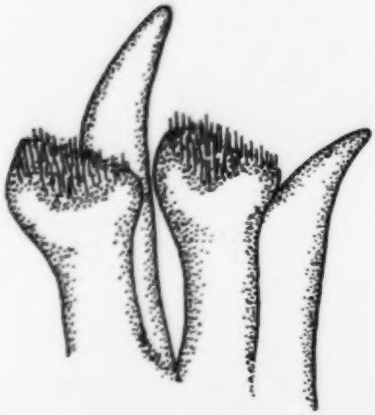

Figure 34. *B. proboscidea*, setae of the modified fifth setiger.

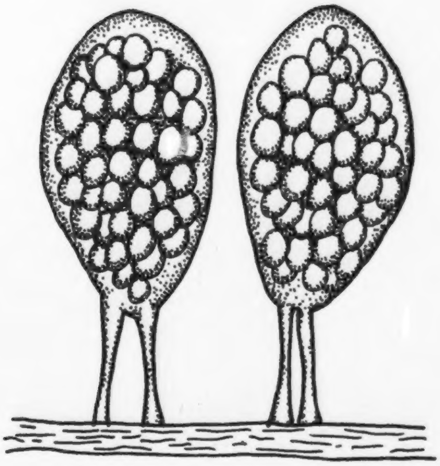

Figure 35. *B. proboscidea,* portion of a string of egg capsules.

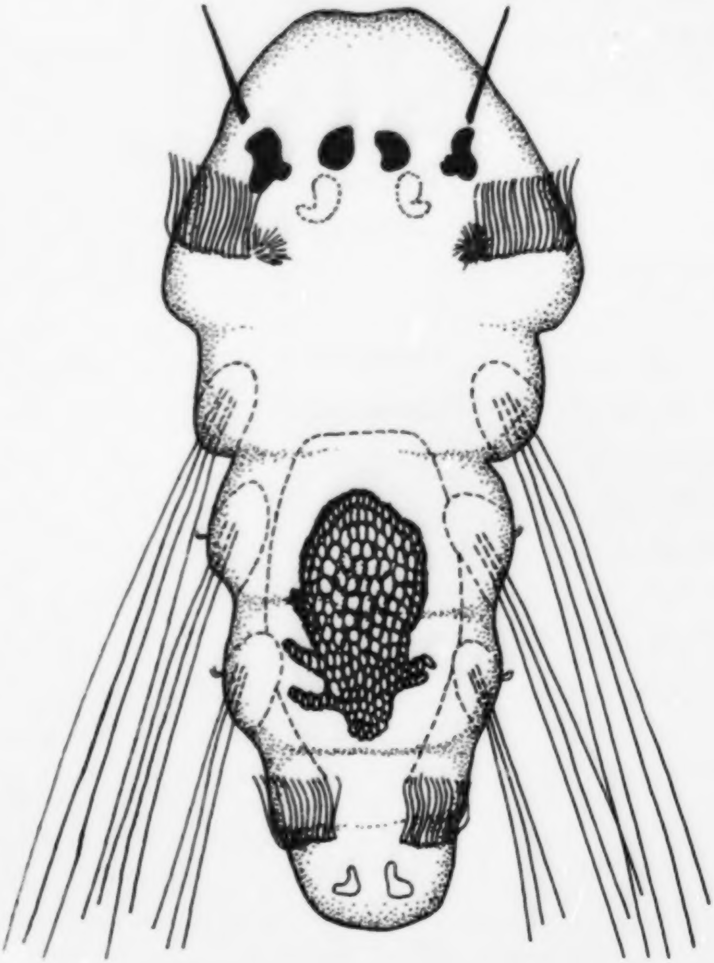

Figure 36. *B. proboscidea*, three setiger larva.

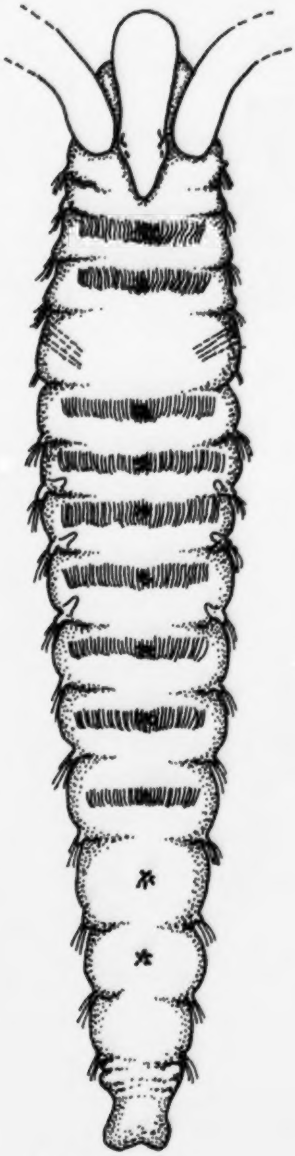

Figure 37. *B. proboscidea*, 15 segmented larva.

APPENDIX G

CULTURE AND BIOASSAY PROCEDURES FOR POLYCHAETOUS ANNELID: *Dinophilus* sp.

CULTURE TECHNIQUES

Equipment and Supplies

A dissecting and compound microscope are required for examination and identification of the archiannelid *Dinophilus* sp. Approximately one m^2 of shelf space is required in order to culture hundreds to thousands of this species per month. A piped compressed air system is the most suitable method of supplying air to aquaria. While *Dinophilus* sp. is quite capable of withstanding broad ranges of temperature, best results have been obtained when the laboratory temperature is maintained at 19±1°C. Consumable supplies include 3.78 ℓ (1 gal) jars with covers, pasteur pipettes, petri dishes, sieves, microporous filter system, air stones, and plastic tubing. A blender is convenient for grinding food into finer particles for *Dinophilus*. Since this polychaete is so small, it is best to pass all seawater through a microporous filter (45 μ) to remove microorganisms. Approximately 50 liters of seawater will be more than adequate to culture this species per month.

Collecting Techniques and Life History

The genus *Dinophilus* sp. is a minute polychaete that has been reported from many regions of the world. Because of its small size, it is probably more common than the previous reports indicate. It is impossible to observe in the field because of its size. The most convenient way to collect *Dinophilus* is to bring clumps of fouling organisms from boat floats or pilings into the laboratory and place them in a pan of seawater. After a period of time, examine the animals that crawl up along the sides of the pan under the microscope and look for *Dinophilus* sp. (Figure 38). Remove with a pasteur pipette, and place in a petri dish containing seawater.

Species of the genus *Dinophilus* are very difficult to identify and require the help of an expert. Thus far, this laboratory population has not yet been identified to species.

Members of the genus *Dinophilus* are dioecious. Eggs are laid in a small capsule and generally number three to four per capsule (Figure 39). Sexual maturity is generally reached within one week. Since this species has such a short life history, it is unnecessary to use special techniques with different stages. Stock colonies can be maintained at 19±1°C with a minimum of time by utilization of 3.78 ℓ (1 gal) jars. Add 2,500 ml microporous-filtered seawater to each container. Provide aeration with an aquarium stone connected to a plastic tube and air supply. The top of the jar can be covered with a plastic top to minimize evaporation. Many specimens (20-50) can be placed in a single gallon jar. Add about 1.0 ml of Tetramin solution (see below) about every seven days. Since this species reproduces rapidly, the population within the aquarium reaches its peak in about two to three weeks then declines. Therefore, each jar should be reestablished about once a month. The reproductive capacity of *Dinophilus* can be slowed by placing the aquarium in a cold bath at 13-17°C.

Food for *Dinophilus* consists of finely-powdered Tetramin that is prepared by grinding the flakes in a blender. The powdered Tetramin is then placed on a 0.06 mm mesh sieve and shaken for a few minutes. Use only the powder that passes through the sieve and collects within the pan. A 1 percent mixture of fine powder to seawater is prepared, and 1.0 ml is pipetted into each aquarium per week.

Problems in Culturing--

Most of the difficulty in culturing *Dinophilus* can be attributed to the lack of regular, systematic care. However, since this species reproduces so rapidly, ten aquaria should be sufficient to meet most laboratory needs. Since the population within an aquarium reaches a peak within two to three weeks then declines, it is important to keep a record of its establishment. A fungal growth occurs whenever the animals are overfed. This fungal growth can lead to a poor yield. If a fungal growth occurs, stir up the material on the bottom and observe the next day. If the fungal growth reappears, reestablish the aquarium.

BIOASSAY PROCEDURES

Equipment and Supplies

The only additional supplies required for conducting bioassays with *Dinophilus* are stender dishes or small plastic petri dishes.

96-Hour Experiments with *Dinophilus*

Because of its rapid life history, 96-hour bioassays may be difficult to conduct since it is possible for this species to have reproduced during this short experimental period. Only the smallest specimens should be used in this experiment. Trial experiments should be carried out to determine whether or not 96-hour tests can be conducted under conditions in the particular laboratory. Assuming it is possible to conduct 96-hour bioassays successfully, pipette material from the bottom of an aquarium into a petri dish and examine for young specimens under the dissecting microscope. Transfer appropriate sized specimens into a second petri dish with a pasteur pipette. Examine all specimens for injuries, and discard those that are not suitable for experimentation. Place five specimens within a stender dish containing 20 ml of seawater, use ten dishes per concentration. A minimum of five concentrations of toxicant plus control should be employed for each test. Since these worms are so small, it is necessary to feed them during the 96-hour experimental period. Add 0.1 ml of food to each container. Count the number of living *Dinophilus* at the end of the experimental period. Discard all specimens at the conclusion of the experiment.

Long-Term Experiment through Reproductive Period

Follow the same procedures outlined for the 96-hour experiment. Generally, the same experiment can serve both as a 96-hour and long-term bioassay. Allow the experiment to extend to 21 days at which time all specimens should be counted. The number of egg capsules containing embryos can be counted and recorded separately.

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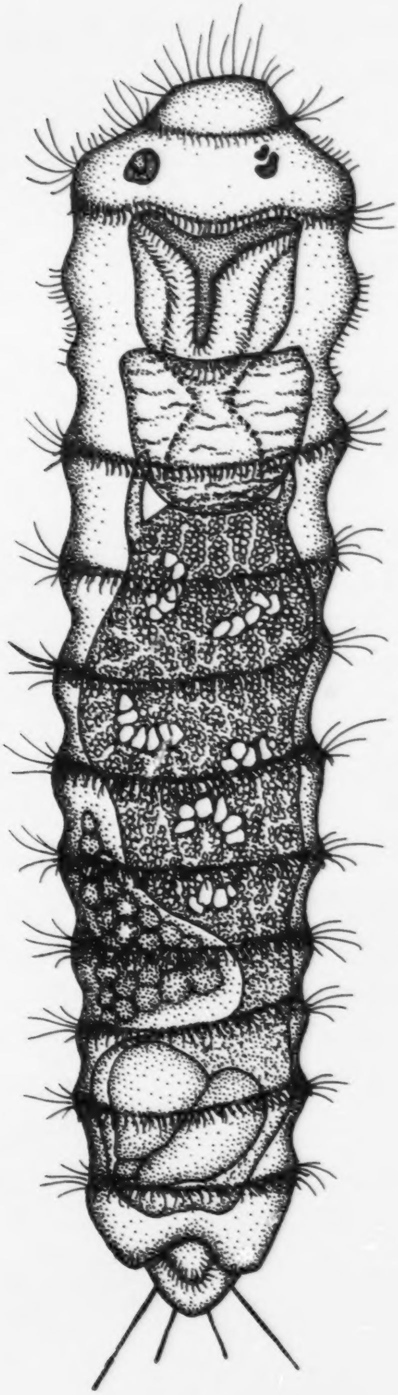

Figure 38. *Dinophilus sp.*, dorsal view of entire worm.

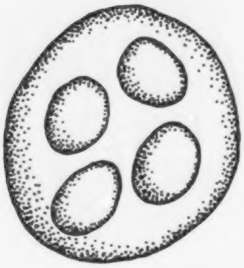

Figure 39. *Dinophilus sp.*, egg capsule.

APPENDIX H

CULTURE AND BIOASSAY PROCEDURES FOR POLYCHAETOUS ANNELIDS: *Cirriformia luxuriosa* and *Cirriformia spirabrancha*

CULTURE TECHNIQUES

Equipment and Supplies

Dissecting and compound microscopes and a blender are the only pieces of equipment required to culture these two species of *Cirriformia*. A minimum of 10-12 m^2 of shelf space is necessary in order to culture populations of 1,000-3,000 specimens each of these two species. A constant temperature laboratory set at 19±°C is an ideal environment for both of these species; however, if time becomes a critical factor, then placing the aquaria in a cold room (or cold bath) at 15-17°C slows the metabolic rate sufficiently to reduce the amount of time necessary for routine maintenance of the populations. Natural, filtered seawater (35 $^{o}/oo$ salinity) is the most satisfactory source of seawater; approximately 5,000 liters of seawater per month is needed to maintain populations of 2,500 specimens of these two species. Because of the frequent feedings and periodic water changes, it will be necessary to spend 15 hours per week to maintain these populations. A central compressed air system is the most satisfactory source of air for *Cirriformia*. Consumable supplies include 3.78 ℓ (1 gal) jars and 56.1 ℓ aquaria, medium grade aquarium sand, forceps, brushes, petri dishes, pasteur pipettes, aquarium stones, plastic tubing, and food supply.

Collecting Techniques and Life History

Both species of *Cirriformia* are known primarily from California, although both species have been reported from the gulf of California. A related species, *C. tentaculta*, found in European waters, is similar, and the techniques described for *C. luxuriosa* and *C. spirabrancha* would probably apply equally well to *C. tentaculata*. Both species were collected from Alamitos Bay in Long Beach but from different localities. Both were taken from fine sediments that generally possessed sulfide odors. The worms live in the upper three to six cm of sediment and can be collected either by hand or by sieving the sediment through a course screen (2.0 mm mesh). *Cirriformia luxuriosa* also occurs within *Mytilus edulis* communities attached to floating boat docks, especially where the water circulation is limited. Because of the possession of numerous gills throughout much of the length of the body, specimens become entangled and are nearly impossible to separate.

The field-collected specimens should be placed in pans with about 1 cm thickness of medium grade aquarium gravel (2.0 mm diameter) and 3-5 cm of seawater. The specimens will burrow into the gravel and become separate from one another. The untangled worms can then be placed in either 3.78 ℓ jars (5 specimens/aquarium) or 56.1 ℓ (15 gal) aquaria (50-100) and provided with one to two cm thickness of gravel, seawater, an air supply, and food.

Living specimens of these two species are easily distinguished from one another by color of the tentacles; *C. luxuriosa* have red tentacles (Figure 40), and *C. spirabrancha* (Figure 41) have green ones. Unfortunately, this color difference disappears upon preservation. Preserved specimens are distinguished by one or rarely two heavy black spines in both the posterior notopodial and neuropodial regions in *C. luxuriosa* (Figure 42) and several yellow spines in both the posterior notopodial and neuropodial regions in *C. spirabrancha* (Figure 43). The differentiation of preserved juvenile specimens is more difficult because the spines in *C. luxuriosa* may not yet be either big or black.

The life cycles of both *Cirriformia luxuriosa* and *C. spirabrancha* are long, apparently two years and one year, respectively, and as a result, only a limited amount of success in culturing this species from egg to egg has been accomplished. Since both of these species are large (lengths up to 16 cm and wet weight up to 4.0 g) and their life cycles long, the potential value of these species lies in long-term studies involving the uptake of toxicants. The sexes are separate and the eggs and sperm are released through pores just anterior to the neurosetae. Both sexes emerge from the sediment to release their gametes onto the surface where fertilization occurs. Females spawn an estimated 100,000 to 500,000 oocytes at one time. Trochophores appear at 16 hours and become elongated (Figure 4) at 2 days. Larval settlement occurs on the fifth day, and feeding commences on the eighth day. Two setigerous segments appear on the eleventh day when the first and second branchial buds are noted (Figure 45). Growth continues at the rate of one new segment per day (Figure 46). Gametes are noted in both species at nine months. Spawning occurred after one year in *C. spirabrancha* but had not yet occurred in *C. luxuriosa* after two years, at which time the colony was destroyed.

A mixture of *Enteromorpha* sp. and Biorell is the most satisfactory food source. Large quantities of the green alga *Enteromorpha* sp. can be collected from estuaries during high tides. The alga is collected by hand and washed at the site to remove as much adhering debris and sediments as possible. The algae is spread out on chicken wire and allowed to air-dry. When it is dry, usually within 24 hours, it is gathered and can be stored for an indefinite period of time. Biorell is a commercially available fish food flake. The mixture of *Enteromorpha* and Biorell is made at the ratio of 4:1 by dry weight. This mixture is pulverized in a blender and shaken through a sieve possessing a 0.06 mm opening. Feed smaller sized worms 0.05 g per worm five times a week. The amount of food should be increased with the growth of the worms. Feed worms over ten cm in length 0.3 g per worm, five times a week. To judge whether the colony of *Cirriformia* is being overfed, examine the condition of the surface of the gravel. If the gravel is kept clean, the worms are not being overfed. Increase the amount of food per feeding until debris begins to accumulate on the surface of the gravel The seawater should be changed about every two weeks at which time the gravel should be rinsed to remove any buildup of organic material.

Problems in Culturing--

Maintaining a colony of *Cirriformia luxuriosa* and *C. spirabrancha* requires daily care. If such care is not given these species, then growth may be slowed and black sulfide deposits may build up, which could lead to death of the specimens. The surface of the gravel within each aquarium should be examined daily prior to feeding to determine whether or not the colony is being underfed or overfed. It is important that the gravel be washed every two weeks to remove the fecal material. The seawater should be changed at this time. There are no known enemies of either species, nor are the species cannibalistic.

TOXICITY TEST PROCEDURES

Equipment and Supplies

Toxcity tests can be conducted in 500 ml Erlenmyer flasks; other supplies required were specified under culture techniques. Stender dishes or small petri dishes are satisfactory containers for larval bioassays.

96-Hour Experiments with Adults

Remove specimens from stock cultures individually and place in separate petri dishes. This procedure eliminates any possibility of the gills of specimens becoming entangled with one another, which would greatly increase the time in setting up a bioassay test. Examine each specimen individually under a dissecting microscope for injuries, and discard such specimens. Place a single worm in a 500 ml Erlenmeyer flask containing 100 ml of toxicant. Close the flask with a silicone stopper. Use 20 worms per concentration and a minimum of five concentrations plus controls. Examine each worm daily for death, and discard all specimens.

96-Hour Experiments with Trochophore Larva

Since both species of *Cirriformia* lay tens of thousands of eggs, many larval toxicity tests can be conducted from the fertilized eggs from one spawning. Draw up ten trochophore larvae with a pasteur pipette, and place in a dish containing seawater and the toxicant; use a minimum of five dishes per conentration and a minimum of five concentrations plus control. No food is required for a 96-hour experiment. Examine at the end of the experimental period for living trochophores, and destroy all specimens at this time. Dead torchophores will decompose within a short period of time and cannot generally be found by the end of the experimental period.

Death in *Cirriformia*--

Death in both species of *Cirriformia* is defined as the absence of movement when the flask is gently rolled. Rupture of the body wall often occurs just prior to death. The body gills of stressed *Cirriformia* are usually fragmented.

Long-Term Experiments with Adults

Since both species of *Cirriformia* are capable of surviving one to two months without feeding, the same experiment can be utilized for both the 96-hour and 28-day bioassay. If the objectives are to conduct a long-term experiment over several months, it will be necessary to provide specimens with food (in the form and amounts specified under the procedures outlined for culturing these worms). Because of the large size of the worm, both of these species may be convenient test organisms for long-term experimentation to study uptake, retention, and metabolism of a toxicant. However, it is important to note that such an experiment has not yet been conducted with either species. If such an experiment is contemplated, the procedures outlined under the section on culturing should be followed. A large number of specimens (25 to 50) could be placed in a 56.1 liter aquarium (Provide approximately one liter of seawater per specimen). Gravel must be provided and the worms fed the required food five times a week. Either species could be used to conduct long-term studies in a flow-through system.

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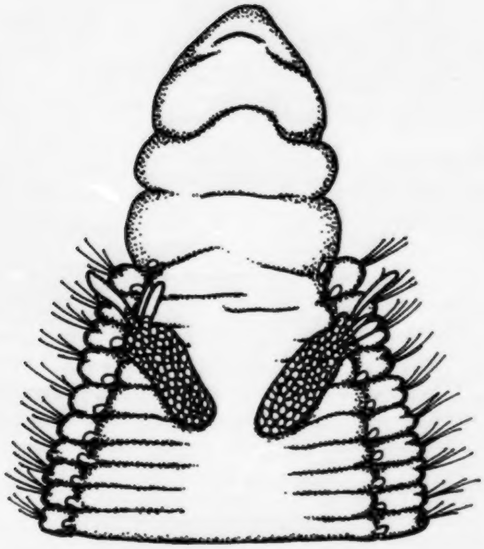

Figure 40. *Cirriformia luxuriosa*, anterior end, dorsal view.

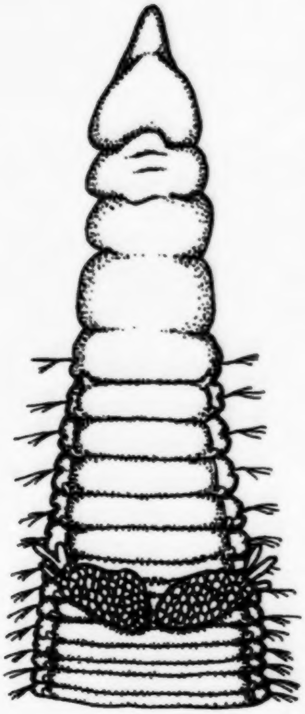

Figure 41. *C. spirabrancha*, anterior end, dorsal view.

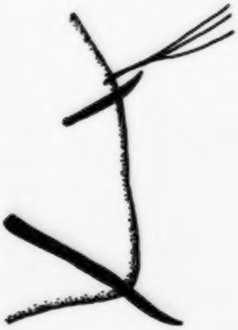

Figure 42. *C. luxuriosa,* outline of segment 150 (after Moore, 1904).

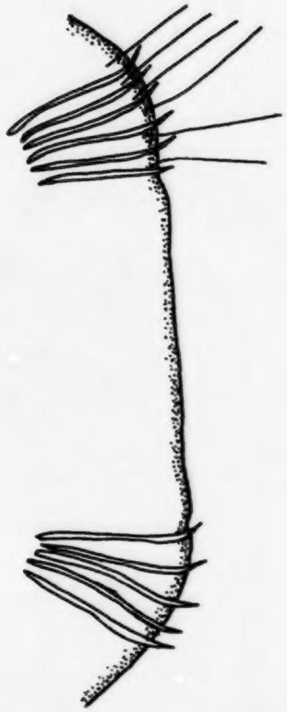

Figure 43. *C. spirabrancha*, outline of segment 150 (after Moore, 1904).

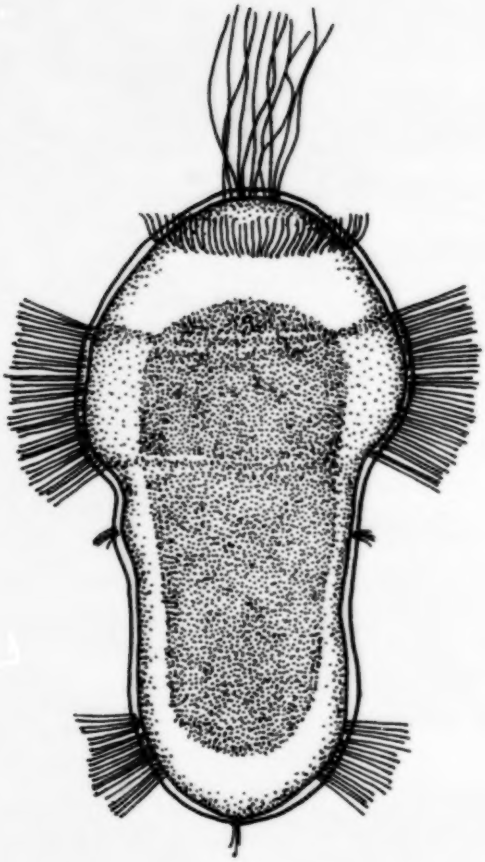

Figure 44. *C. luxuriosa*, late trochophore stage.

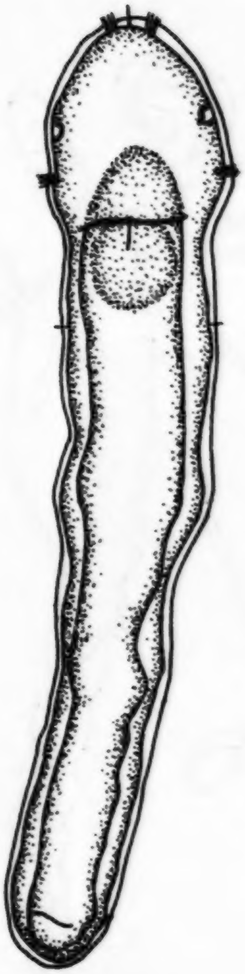

Figure 45. *C. luxuriosa,* metamorphosed juvenile.

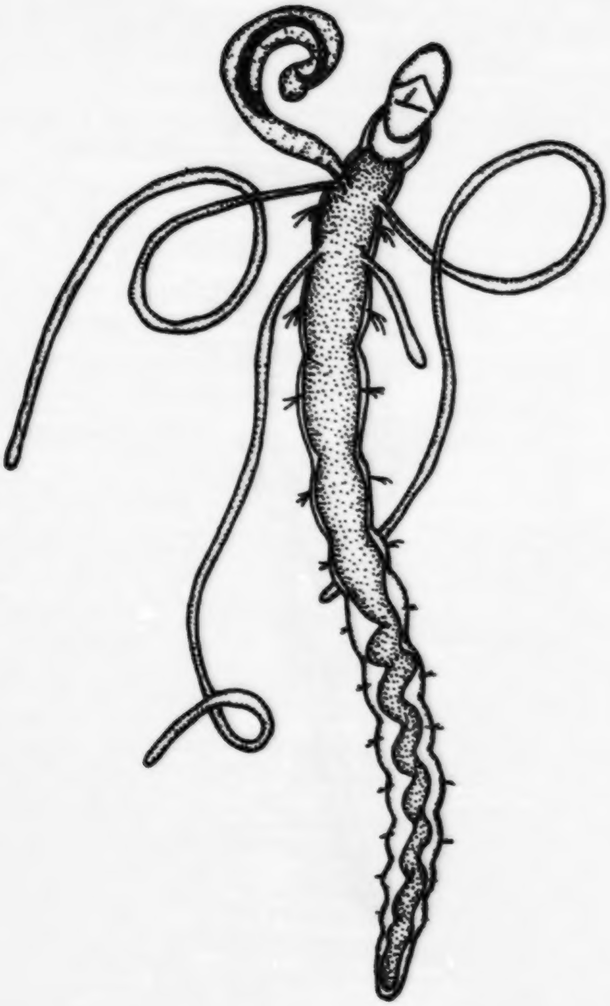

Figure 46. *C. luxuriosa*, late juvenile stage.

APPENDIX I

CULTURE AND BIOASSAY PROCEDURES FOR POLYCHAETOUS ANNELID: *Halosydna johnsoni*

CULTURE TECHNIQUES

Equipment and Supplies

A dissecting and compound microscope are needed for observation of the various stages in the life history of *Halosydna johnsoni*. Some method of maintaining a temperature of 18°C (constant temperature room or water bath) is needed. A cold bath measuring 1.2 x 2.4 m will provide sufficient space to culture and maintain approximately 500 adults in petri dishes, which are placed in enamel pans and 25 3.78 ℓ jars containing juveniles. Since each adult must be fed individually twice a week, approximately ten hours a week is necessary to maintain a population of *Halosydna* of this size. Supplies required include petri dishes, glass tubing, aquarium stones, plastic tubing, plankton netting (Nitex 7 μ), pasteur pipettes, microporous filter system, brushes, 25 x 35 cm trays, forceps, and screens with 0.147 and 0.06 mm openings. Food consists of frozen brine shrimp, a mixture of Biorell, Tetramin, and alfalfa powder, and a *Dunaliella* culture.

Collecting Techniques and Life History

Halosydna johnsoni is the most commonly occurring scale worm in southern California (Figure 47). A closely related species, *H. brevisetosa*, is found in colder waters of the eastern Pacific Ocean; however, the distribution of the two species can overlap. These two species differ primarily in the type of neurosetae. Some question of the validity of the two species has been raised, since it has been demonstrated experimentally that each "species" can develop the other "species" setal type in warm or cold temperatures (Hillger and Reish, 1970). Since all specimens reared from fertilized eggs to adults developed only bifid neurosetaes, the specific name *H. johnsoni* is retained herein as the valid name for this population with the realization that subsequent studies may not substantiate this conclusion.

Halosydna is most easily collected from the fouling community attached to floating boat docks since collections can be made independent of tidal conditions. Clumps of mussels (*Mytilus edulis*) and other organisms are removed from the dock float and placed in shallow pans. Separate this material to free the worms. Remove the worms from the pan with a brush, and place in a separate container for transport to the laboratory. In the laboratory, transfer the specimens to individual petri dishes. Place the dishes in trays in an 18°C cold water bath. Two to three days later place a piece of glass tubing, which is of the appropriate length and diameter for the given worm, in each petri dish. The worm is maneuvered into the tubing with a small brush. If the worm attempts to vacate the tube, the protruding end is tapped lightly. This process is repeated until the worm accepts the tube as its new home. One week after collection the worms are fed a few frozen brine shrimp. For the first few feedings it is necessary to grasp several brine shrimp with a pair of forceps and wiggle them near the anterior end of the worm to initiate a feeding response. After three to five feedings most specimens are trained sufficiently to accept the food so that it is only necessary to place the brine shrimp in the vicinity of the anterior end. The worms are fed until satiated twice a week. Change the seawater after each feeding.

The sexes are separate and can only be distinguished after development of the gametes. To distinguish one sex from the other, examine the ventral surface; females appear olive-green because of the presence of maturing ova, and the males are white as the result of maturing sperm. Spawning is induced by transferring mature specimens from 18°C to a petri dish containing water with a temperature of 20 to 25°C. Actual release of sperm has not been observed, but all gametes are not released at the same time. Females release their ova through paired ventrally situated nephridial papillae. Initially, the ova are released individually at a more or less staccato rate that then becomes a steady emission followed again by the staccato rate of release. Nearly all ova are released at one spawning. Up to an estimated 240,000 ova are produced by one female; the ova are small, flattened somewhat, and measure 88 to 94 µ long and 40 µ thick. Gametes of both sexes are transferred from petri dishes to a 3.78 ℓ jar containing 18°C seawater; the water is stirred intermittently over a 15-minute period. The jar is filled with seawater and divided into three additional jars that in turn are filled with 18°C seawater. Place the jar in an 18°C cold bath and provide it with aeration. In order to measure the success of fertilization, remove the jar from the cold bath and place a strong light at the top to attract the trochophore larvae (Figure 48). The density of the trochophores at the surface is noted as well as the number of unfertilized eggs or undeveloped zygotes adhering to the bottom of the aquarium. That portion of the water containing trochophores is decanted, diluted two to three times, and poured into the appropriate number of jars; the amount of dilution is dependent upon the density of the trochophores in the initial jars. After dilution, place the jars in the cold bath at 18°C and provide aeration. At six days, 60 ml of *Dunaliella tertiolecta* culture are added to each aquarium followed by an additional 20 ml of culture every five days for three times. The majority of the larvae metamorphose (Figure 49) at about 20 to 25 days and settle to the bottom. Do not feed between days 25 to 40 at which time the contents of the jar are filtered through a 7 µ nitex plankton netting. The netting is placed in a petri dish containing seawater and examined under a dissecting microscope for presence of larvae. Transfer the young *Halosydna* with an appropriate sized pasteur pipette and place in a clear 3.78 ℓ aquaria containing 3:1 of millipore-filtered seawater and 0.6 g of a mixture of two parts (dry weight) of alfalfa powder, one part of Biorell powder, and one part Tetramin powder. A maximum of 50 larvae (Figure 50) can be placed in each jar, which is then placed in the 18°C cold bath. After 60 to 70 days of development, specimens with 25 or more segments can be removed and placed in individual petri dishes and fed brine shrimp as described for maintaining adults. Sexual maturity is reached after nine months of growth.

Problems in Culturing--

Since the adults are aggressive and cannibalistic, they must be kept in separate containers. In addition, each one must be fed individually with frozen brine shrimp. The individual personal attention required by each adult necessitates a considerable amount of time. If the proper attention and care are not given the adults, death can follow quite rapidly because of fouling of the water. As typical for most species having pelagic larvae, many larvae fail to metamorphose. Undoubtedly, if more time and attention were spent caring for the culture a higher percentage of the larvae could survive.

TOXICITY TEST

Equipment and Supplies

No additional equipment and supplies are required to carry out bioassays other than those specified above.

96-Hour Experiments with Adults

Place 50 ml of seawater and toxicant in a ten cm diameter petri dish. Transfer each *H. johnsoni* in its glass tubing to a petri dish containing clean seawater, and examine for injuries under the dissecting microscope. Then transfer all healthy specimens to the experimental container. Use 20 worms per concentration with a minimum of five concentrations plus control for each bioassay. Do not feed the animals during the course of the experiment. Examine for deaths daily, and discard all specimens at the conclusion of the experiment.

Long-term Experiment with Adults (28 Days)

Set up the experiment the same as the 96-hour test; in fact, the same experiment can serve as both by following the procedures given below. After examination for deaths at 96 hours feed each animal with frozen brine shrimp as indicated under the techniques of culturing the adults. After feeding, renew the seawater and toxicant; feed twice a week with water changes made after each feeding.

96-Hour Experiments with Trochophore Larvae

The trochophore larvae of *H. johnsoni* are sensitive and especially useful for 96-hour tests. Separate out the trochophore larva from the top of the aquarium as specified above. Pipette larvae into a petri dish containing seawater. Use ten larvae per dish with ten petri dishes per concentration and five test concentrations plus control. Count the number of living trochophores at the end of 96 hours and discard all specimens at the conclusion of the experiment.

Death in *Halosydna*--

Stressed adult *Halosydna* will usually leave the glass tubing and may or may not exhibit erratic movements such as turning over and over. Specimens under severe stress, such as those specimens placed in a highly toxic solution, may go into convulsions and fragment at the mid-body region. Death follows rapidly in this case. Specimens in a solution at or near the calculated LC_{50} will generally leave the tube, leytrae (scales) will be cast off, and dorsal cirri may fragment. These specimens may live beyond a 28-day period, but they will not feed.

Dead trochophore larvae are often difficult to find because decomposition is so rapid in these young worms. It is much more convenient to count living specimens. Trochophore larvae are considered dead if they fail to move through the water mass and if there is an absence of ciliary movement. A stressed trochophore is generally more or less motionless, but its cilia will continue to move at a reduced rate.

Use of *Halosydna johnsoni* in Bioassays

Halosydna johnsoni has only been utilized to a limited extent in bioassays. The advantages of this species as a test organism are the ease in obtaining a large number of trochophore larvae, the sensitivity of both larvae and adults to many toxicants, and the large size of adults that permits body burden analyses of individual specimens. The primary disadvantage of this species is the considerable amount of time that must be spent in maintaining a relatively small population.

LABORATORY CULTURE OF *Dunaliella tertiolecta*

Medium

Seawater: Use about 30-32 °/oo salinity seawater (90% seawater and 10% double distilled water). Filter through a microporous-filter system (0.45 µ). For 50 ml quantities, mix complete medium (below) and autoclave at 15 psi for 15 minutes. For larger quantities autoclave phosphate separately and then add to the medium. If a serious precipitation occurs, it may be necessary to filter sterilized medium prior to use.

Major nutrients: Nitrate--5 g KNO_3 made to 1,000 ml with H_2O. This stock solution may be stored in a refrigerator for up to four months. Add 1.0 ml stock solution to one liter distilled water to make a final solution; use ten ml of this solution per liter of culture medium. Do not store final solution.

Phosphate--0.68 g KH_2PO_4 made to 1,000 ml with H_2O. This solution may be stored in a refrigerator for up to four months. Add 1.0 ml stock solution to one liter distilled water to make a final solution; then ten ml of this solution per liter of culture medium. Do not store final solution.

Minor trace metals-- Stock metals--30 mg $ZnSO_4 \cdot 7H_2O$, plus 25 mg

$CuSO_4 \cdot 5H_2O$, plus 20 mg $CuSO_4 \cdot 7H_2O$ dissolved in 1,000 ml of distilled water.

Iron + Manganese--Dissolve 5.0 g $FeCl_3 \cdot 6H_2O$ and 2.0 g $MnSO_4 \cdot H_2O$kn 1,000 ml distilled water (ignore slight precipitate).

Sodium Molybdate--25 mg $Na_2MoO_4 \cdot 2H_2O$ in 1,000 ml of distilled water.

Sodium Ethylenediaminetetraacetate--Dissolve 50 g of Na_2E.D.T.A.$\cdot 2H_2O$ in 1,000 ml of distilled water.

Metal Mixture--Add 100 ml of the Na_2E.D.T.A.$\cdot 2H_2O$ solution and ten ml each of the three minor trace metal solutions listed above to about 800 ml of distilled water. Adjust the pH to 7.5 with dilute NaOH solution and make up to 1,000 ml with distilled water. Add 1.0 ml to each liter of culture medium. This solution may be stored for up to four months in a refrigerator.

Vitamins:Vitamin B_{12}--Dissolve 10.0 mg crystalline vitamin B_{12} in 100 ml of distilled water. Store in deep freeze until use. Immediately prior to use, thaw the solution, remove 1.0 ml, and refreeze the stock solution. Dilute the ten ml of vitamin B_{12} solution with 99 ml of distilled water. Add 1.0 ml of diluted solution to each liter of seawater and then throw away remainder.

Biotin--Dissolve 10.0 mg biotin in 100 ml of distilled water and freeze. Immediately prior to use thaw the solution, remove 1.0 ml, and refreeze the stock solution. Dilute the 1.0 ml with 99 ml distillled water. Add 1.0 ml of diluted solution to each liter of seawater and throw away the remainder.

Thiamine hydrochloride--Dissolve 100 mg of thiamine hydrochloride in 100 ml of distilled water and freeze. Immediately prior to use thaw, remove 1.0 ml, and refreeze the stock solution. Dilute the 1.0 ml with 99 ml of distilled water. Add 1.0 ml of diluted solution to each liter of seawater and throw away the remainder.

Culture

Place 5 to 15 ml of *Dunaliella* from a previous culture in a 500 ml Erlenmeyer flask containing 500 ml of autoclaved medium. The amount of *Dunaliella* depends upon its concentration in the previous culture. Place stoppered Erlenmeyer flasks under grow-lux florescent lights. Depending upon the amount of *Dunaliella* required, the alga can be exposed to continuous light or to a light-dark cycle, which is maintained with an electrical timer. The growth rate can be slowed, if desired, by placing the cultures in a cold bath (17°C) with the lights placed overhead.

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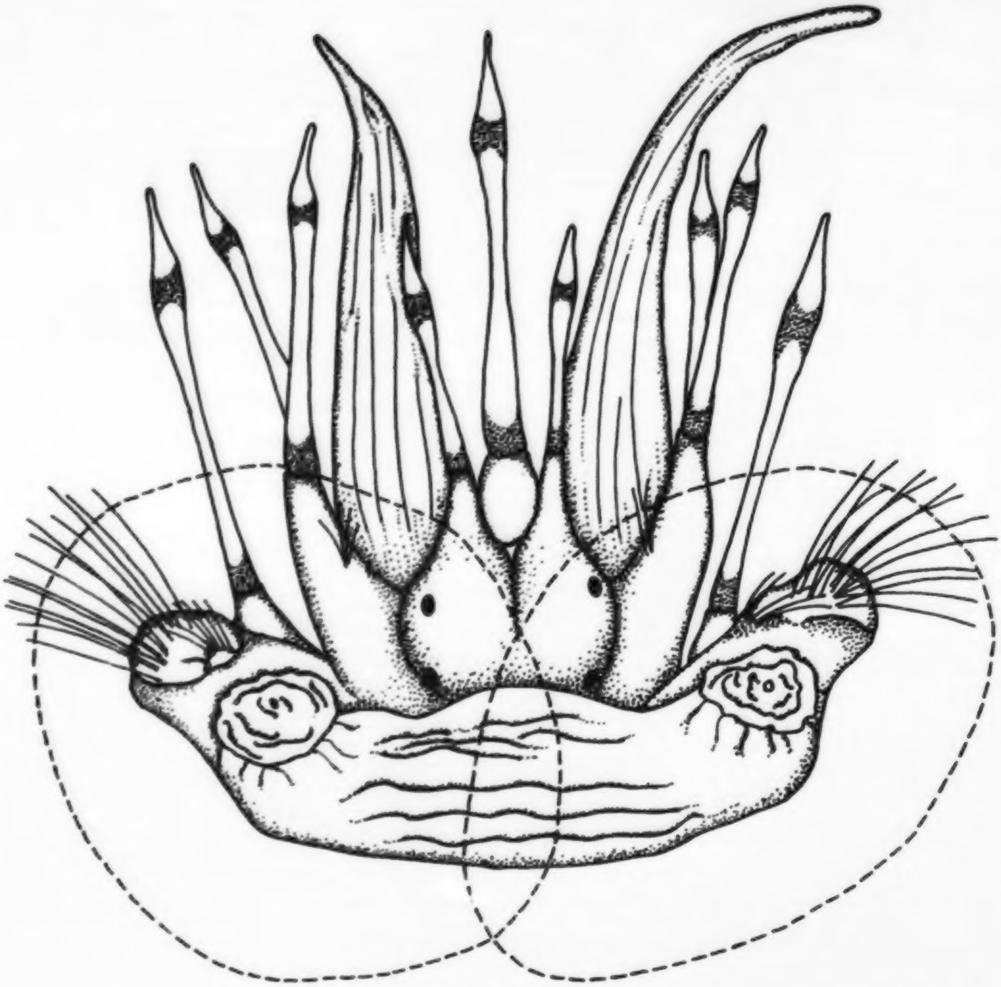

Figure 47. *H. johnsoni*, anterior end, dorsal view.

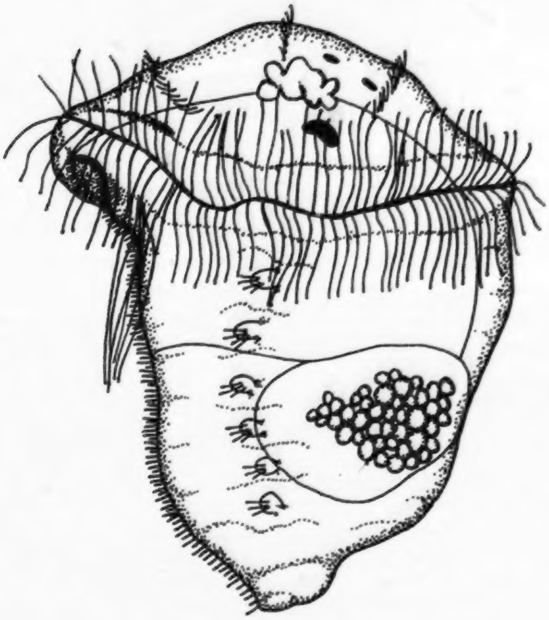

Figure 48. *H. johnsoni*, trochophore larva.

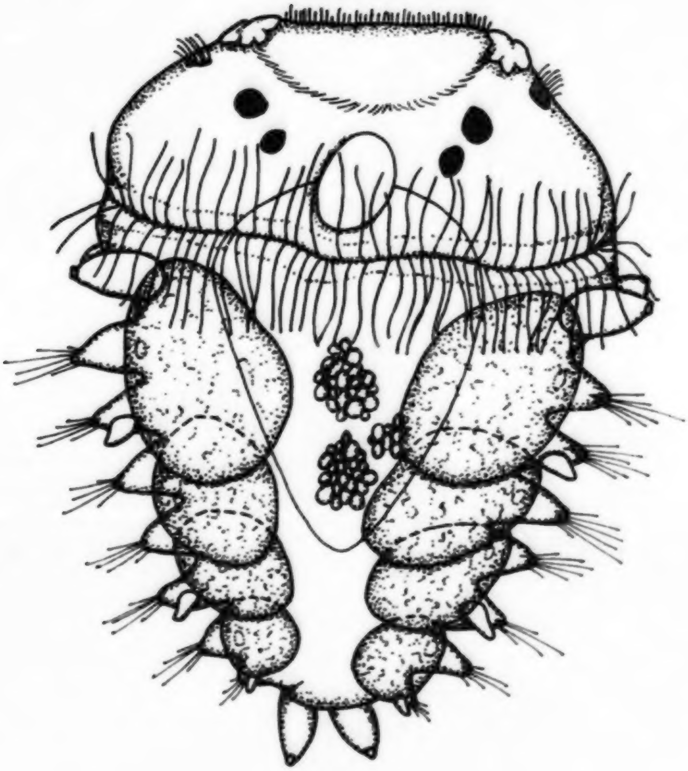

Figure 49. *H. johnsoni*, metatrochophore larva.

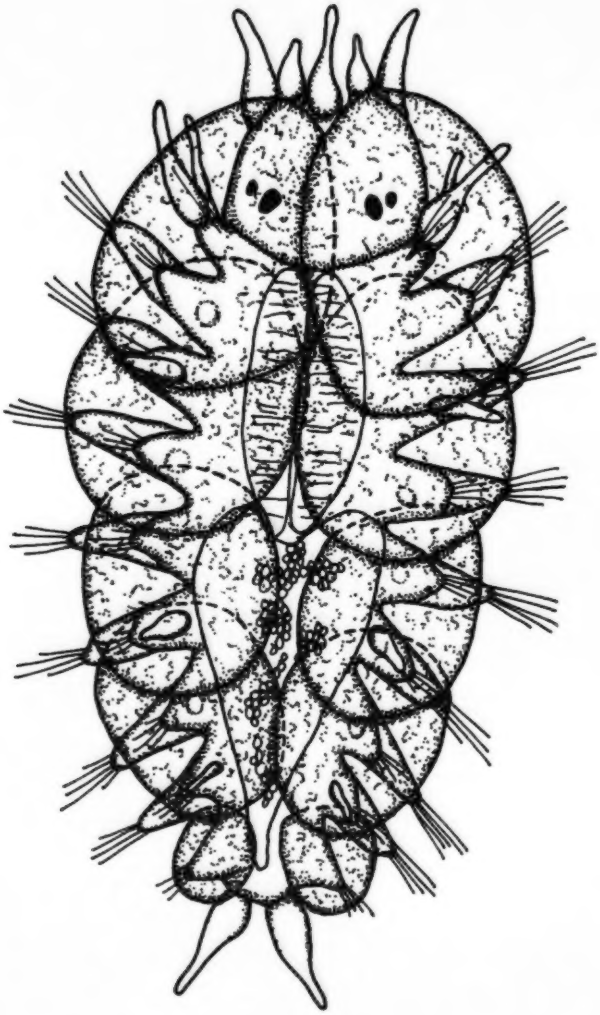

Figure 50. *H. johnsoni*, metamorphosed juvenile.

APPENDIX J

LIST OF PUBLICATIONS AND THESES SUPPORTED BY THIS RESEARCH GRANT

PUBLICATIONS

Reish, D. J., J. M. Martin, F. M. Piltz, and J. Q. Word. 1974. The Induction of Abnormal Polychaete Larvae by Heavy Metals. Marine Pollution Bulletin, 5:125-126.

Oshida, P. S., and D. J. Reish. 1974. The Effect of Various Water Temperatures on the Survival and Reproduction in Polychaetous Annelids: Preliminary Report, Marine Studies of the San Pedro Bay, Calif. Part 3. Allan Hancock Foundation, pp. 63-77.

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Carr, R. S. 1976. The Effects of Petrochemicals on Five Species of Polychaetous Annelids, *Ctenodrilus serratus*, *Ophryotrocha* sp., *Ophryotrocha puerilis*, *Capitella capitata*, and *Cirriformia spirabrancha.* Master's Thesis, Calif. State Univ., Long Beach. 92 pp.

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APPENDIX K

List of Laboratories Which Have Utilized Specimens Cultured in California State University, Long Beach

Many laboratories have obtained specimens or subcultures of polychaetes from this laboratory for use in their own studies. One valuable spin-off from developing the techniques for culturing polychaetes through complete life cycles and establishing laboratory inbred colonies is that it makes it possible for others to conduct studies which would otherwise not have been possible. Specimens have been sent to the following laboratories through 1979.

1. Environmental Research Laboratory, Narragansett, Rhode Island (Bioassays with metals).
   Capitella capitata
   Cirriformia luxuriosa
   Ctenodrilus serratus
   Neanthes arenaceodentata

2. U.S. Navy, Anapolis, Maryland (Bioassays with dredge spoils).
   Neanthes arenaceodentata

3. U.S. Navy, San Diego, California (Bioassay with dredge spoils).
   Neanthes arenaceodentata

4. U.S. Army Corps of Engineers, Vicksberg, Mississippi (Bioassays).
   Neanthes arenaceodentata

5. Texas A&M University (Bioassays with petroleum hydrocarbons).
   Capitella capitata
   Neanthes arenoceodentata

6. Scripps Institute of Oceanography (Bioassays with petroleum hydrocarbons).
   Capitella capitata
   Neanthes arenaceodentata

7. Allan Hancock Foundation, University of Southern California (Bioassays with many different types of toxicants).
   Neanthes arenaceodentata
   Ophryotrocha diadema

8. School of Medicine, University of California Los Angeles (Medical research with eggs).
Neanthes arenaceodentata

9. University of California, Irvine, California (polychaete metabolism).
Neanthes arenaceodentata

10. University of Alberta, Calgary, Alberta, Canada (trematod life cycles).
Neanthes arenaceodentata

11. McGill University, Montreal, Quebec, Canada (experimental studies with eggs).
Capitella capitata

12. Station Marine d'Endoume, Marseille, France (Bioassays with metals).
Capitella capitata

13. Southern California Coastal Water Research Project, El Segundo, California (Bioassays).
Ctenodrilus serratus
Neanthes arenaceodentata

14. Orange County Sanitation District, Fountain Valley, California (Bioassays).
Neanthes arenaceodentata

15. NUS Corporation, Houston, Texas (Bioassays).
Neanthes arenaceodentata

16. EG&G, Bionomics, Pensicola, Florida (Bioassays).
Neanthes arenaceodentata

17. Lockheed Corporation, Carlsbad, California (Bioassays).
Neanthes arenaceodentata

18. Marine Biological Consultants, Inc., Costa Mesa, California (Bioassays).
Capitella capitata
Ctenodrilus serratus
Neanthes arenaceodentata
Ophryotrocha diadema

19. Batelle Northwest Laboratories, Washington (Bioassays with petroleum hydrocarbons).
Neanthes arenaceodentata